Cloning of a Gene for X-linked Deafness (DFN3)

&

Cloning of a Candidate Gene for X-linked Mental Retardation

S.M. van der Maarel

Cloning of a Gene for X-linked Deafness (DFN3)

Cloning of a Candidate Gene for X-linked Mental Retardation

Cloning of a Gene for X-linked Deafness (DFN3)

Cloning of a Candidate Gene for X-linked Mental Retardation

Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor
aan de Katholieke Universiteit Nijmegen,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op
dinsdag 18 februari 1997
des namiddags om 1.30 uur precies

door

Silvère Maria van der Maarel
geboren op 9 april 1967
te Beverwijk

Promotor	Prof dr V C H H Ropers
Co-promotor	dr F P M Cremers
Thesis committee	Prof dr B Wieringa
	Prof dr R N H Konings
	Prof dr G W A M Padberg

ISBN 90-9010279-5
Druk Benda BV, Nijmegen

voor Pauline en voor mijn ouders,
ter nagedachtenis aan mijn grootvader

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION AND AIM OF THIS THESIS

1. General Introduction

1.1 Strategies for the Elucidation of Hereditary Disorders

All human medical conditions, perhaps even self inflicted trauma, may have a genetic component These genetic components may vary from single gene defects, resulting in monogenic patterns of inheritance, to many genes interacting in a complex way as in polygenic and multifactorial disorders Until now, efforts to identify genetic factors underlying inherited disease have focused on monogenic traits of which by now over 500 (1) have been regionally mapped or elucidated at the molecular level, and for most of the common monogenic disorders, the underlying gene defect is known

There are three main strategies to uncover the nature of these disease genes (2) During ***functional cloning***, the information on the biochemical defect or the structure of the corresponding protein is the key for the gene identification Linus Pauling for example, correctly postulated that a molecular abnormality of the hemoglobin protein was the cause for sickle cell anemia (3) Subsequent isolation and sequencing of the protein eventually led to the actual cloning of the underlying gene defect (4) Unfortunately, our knowledge on the fundamental biochemical pathways involved in complex issues such as cell-cell interactions, cellular signalling, and development is very limited and hampers the large scale routine application of this strategy

Positional cloning is based on the chromosomal localization of the gene, i e the position of the gene in the human genome In general, this information can be obtained through linkage analyses in affected families However, in approximately 60% of the genes cloned in this way, cytogenetic rearrangements such as deletions or translocations have facilitated the precise mapping of the gene, and in many more, submicroscopic deletions have aided their identification (2) Positional cloning was first successfully applied in 1986 when the gene for X-linked chronic granulomatous disease was isolated (5) Another major breakthrough was the cloning of the gene for cystic fibrosis (CF) on chromosome 7, the most common autosomal recessive disorder of Caucasians, which was achieved without the help of chromosome rearrangements (6) At present, over 60 genes have been identified in this way and the number is still growing

Very often, however, the available mapping information is insufficient on its own to allow the isolation of disease genes by positional cloning, and only rarely, the function of these genes can be directly inferred from the clinical phenotype In the majority of cases therefore, positional and functional information has to be combined in order to define promising candidate genes, whose identity has to be confirmed by subsequent mutation screening in (unrelated) patients As the number of cloned, mapped, and sequenced transcripts is rapidly increasing, such positional and/or functional ***candidate gene*** approaches will become more successful These disease genes will help clinical geneticists to understand pathogenic pathways and help molecular geneticists to isolate related genes This, in turn, will enhance the number of successful 'educated guesses' for related disorders For example, the availability of the gene coding for the luteinizing

hormone (LH) receptor enabled the rapid testing and verification of the hypothesis that an autonomously activated g-coupling of the LH receptor might explain the overproduction of testosterone in the absence of stimulation by LH in patients with familial male precocious puberty (testotoxicosis) (7 8) Soon afterwards, the same authors found that loss of function mutations in this gene lead to male pseudohermaphroditism with Leydig cell hypoplasia (9,10)

Two criteria have to be met to successfully apply the latter method First, the linkage interval must represent a rather small segment of the chromosome and secondly, most or all genes that map to this interval must be known To meet the first criterion, the development of genome-wide human linkage maps with markers at an average density of 0 7 centiMorgan (cM) (11) has greatly improved the accurate mapping of disease genes to intervals of 0,5-5cM Moreover, projects aiming at the large scale generation of expressed sequence tags (ESTs), partial DNA sequences of expressed genes, has already led to the identification of 87,983 distinct sequences (12,13) However, without information about the exact map position, these ESTs will be of little help These ESTs, the rapidly evolving Yeast Artificial Chromosome *(YAC a vector system in which up to 2Mb of genomic DNA can be cloned and propagated in yeast cells (14))* maps of the human genome (15), and the initiatives of the Human Genome Project to entirely sequence the human genome before the year 2005, will finally reveal all human genes and greatly facilitate the positional candidate approach One of the most stunning examples of this approach is the identification of 4 genes involved in DNA mismatch repair that had been implied in hereditary non-polyposis colon cancer, where linkage to chromosome 2p and phenotypic information about DNA instability in colon cancers from affected patients was instrumental for the identification of these four genes (16-18)

So far, almost all approaches mentioned above have been applied to single gene disorders However, many of the most frequent medical conditions like hypertension, diabetes, asthma, schizophrenia, neural tube defects, cleft lip and palate, and manic depression, show a murkier pattern of inheritance The genetic dissection of these complex traits, i e all medical conditions that do not show a Mendelian pattern of inheritance, will challenge geneticists for many years to come

1 2 Chromosome Rearrangements and the Positional Cloning of Disease Genes

Linkage analysis is a universal strategy to map genes underlying specific disease phenotypes that segregate in families, but the resolution of linkage mapping is mostly limited by the number and size of the families available Frequently, therefore, these studies leave us with wide linkage intervals Particularly, this holds true for heterogeneous disorders where linkage data from different families cannot be pooled

Balanced chromosomal rearrangements such as translocations and inversions have greatly facilitated the identification of disease-associated genes (2) *De novo* balanced chromosome abnormalities have an incidence of approximately 1 3000 newborns, and 6% of these rearrangements are associated with disease (19) Comparison with the incidence of conspicuous phenotypic alterations in the newborn population suggests that in 50% of the patients with disease associated balanced chromosome rearrangements, the aberrant clinical phenotype and the karyotypic changes are causally related (20) Although in balanced chromosome rearrangements no genetic material is lost, they will give rise to disease phenotypes if chromosome breakage hits a gene for which two functional gene copies are required (20) Disease phenotypes may also arise if the other allele happens to be mutated ('unmasking of heterozygosity' (21)), if the rearrangement results in dominant mutations as in several collagen disorders and human neoplasias, respectively (22,23), or if there is only one functional gene copy as for imprinted (24-27) and X (or Y) chromosomal genes (*chapter 2 3*) In fact, the presence of several X chromosomal breakpoints in females with Duchenne Muscular Dystrophy (DMD) carrying balanced X,autosome (X,a) translocations were among the first mutations described for DMD and their co-localization greatly facilitated the isolation of the dystrophin gene (28) Also, specific balanced translocations have been implicated in human neoplasias The majority of patients with chronic myeloid leukemia (CML) carry a balanced reciprocal translocation between the chromosomes 9 and 22, t(9,22)(q34,q11), which was instrumental for the identification of the molecular mechanisms underlying CML (29,30) This translocation links the oncogene *c ABL* at 9q34 proximal to the *BCR* gene on chromosome 22 resulting in a fusion protein which gives rise to the malignant process (31,32)

Unbalanced chromosome rearrangements such as deletions and duplications are also valuable tools for the identification of disease-associated genes Many of the microscopic or submicroscopic deletions affect more than one gene thereby giving rise to complex contiguous gene syndromes (CGS) The clinical and molecular characterization of partially overlapping CGSs allows the genetic dissection of the complex CGSs into specific subtraits (33) For example, the characterization of various deletions and translocations involving the 11p13 region allowed the identification of several genes that can explain the complexity of the WAGR syndrome, including genes for Wilms' tumour (*WT1*), aniridia (*AN2 / PAX6*), genitourinary malformations, and mental retardation (34-39) Duplications can give rise to CGSs as a result of dosage enhancement of the genes involved Dosage sensitivity is best illustrated by inherited peripheral neuropathies (reviewed in 40) Charcot-Marie-Tooth disease type 1A (CMT1A) is an autosomal dominant peripheral neuropathy associated with a 1,5 Mb tandem duplication at 17p11 2-p12 (41,42) This duplication is the result of an unequal cross-over mediated by flanking repeat sequences (43) The reciprocal deletion of this duplication resulting from the same unequal cross-over, has been implicated in the milder autosomal dominant hereditary neuropathy with liability to pressure palsies (HNPP) (44,45) Moreover, point mutations in the peripheral myelin

protein 22 (*PMP22*) gene which maps to this 1,5 Mb duplication have been identified in other families with CMT1(46,47). These results strongly suggests that the *PMP22* gene is (one of) the relevant dosage-sensitive gene(s) in this 1,5 Mb duplication.

To generate a disease phenotype, the rearrangement does not necessarily have to affect the gene itself, but the translocation of genes to a 'wrong' chromosomal environment may also disturb proper gene expression. This phenomenon, named position effect variegation (PEV), was first discovered in *D melanogaster* (48). Schultz showed that when genes were moved from euchromatic regions of the chromosomes to regions of centric heterochromatin by inversions or translocations, these genes lost their activity in some cells but not in others (49). However, when the gene was relocated to its proper position, it regained its normal transcriptional activity. Failure of gene expression by PEV can be explained in two ways. First, the packaging of DNA may extend from the heterochromatic region into the relocated euchromatic gene. Alternatively, genes placed near heterochromatic regions do not complete replication until late S phase, and by that time transcription factors are already bound up by genes in earlier replicating euchromatin (50). Several observations suggest that position effects exist in humans, too. Campomelic dysplasia, a skeletal malformation syndrome with autosomal sex reversal is caused by mutations in the *SOX9* gene on chromosome 17q25, and by translocations located over 50kb upstream of the *SOX9* gene (51-53). Similarly, aniridia can be caused by translocations 85kb upstream from the *PAX6* gene (54), and position effects may also play a role in some patients with the most frequent form of X-linked deafness, DFN3, as outlined in *chapter 7* of this thesis. Moreover, very recently a translocation has been identified that juxtaposes an IgH enhancer approximately 2 kb upstream of the first *PAX 5* exon in diffuse large-cell lymphomas (55). In mice, the first examples of PEV have been observed in transgenic animals (56), as for example in the position dependent variegation of globin transgene expression (57). Disruptions of the zinc finger gene *GLI3* have been identified in patients with the rare autosomal dominant Greig cephalopolysyndactily syndrome (GCPS) (58). Deletions in the mouse homologue *Gli3* are associated with the *Extra toes* (*Xt* and Xt^J) phenotype (59) whereas *anterior digit deformation* (*add*), a mild recessive allelic form of *Xt*, has a complicated transgene integration and a deletion approximately 40kb upstream of *Gli3* (60). Also, deletions outside the testis determining region were found in sex-reversed XY female mice (61). These deletions position *Sry* closer to the Y centromere which is in agreement with the hypothesis that the silencing of *Sry* activity is due to a position effect and does not affect the *Sry* gene itself (62). The recently identified chromosomal rearrangements in the mouse models *Steel-panda* (Sl^{pan}) and *Steel-contrasted* (Sl^{con}) >100kb upstream of the *Steel* (*Sl*) locus are a particularly well studied example (63). The *Sl* locus encodes the mast cell growth factor (*Mgf*) which is a ligand for *c-kit*, a receptor tyrosine kinase, and is essential for the development of germ cells, hematopoietic cells, and melanocytes. Female Sl^{pan} and Sl^{con} mice are sterile whereas the fertility of the males of the same genotype is unaffected. In

these mice, chromosomal rearrangements 115kb and 195kb upstream of the *Mgf* locus, result in tissue specific up- or down-regulation of *Mgf* mRNA expression The fact that the two rearrangements are 80kb apart, result in positive and negative regulation of *Mgf* expression, and that neither of the two alleles could be complemented by other *Sl* mutant alleles (which excludes the involvement of another closely linked gene), strongly suggests that the Sl^{pan} and Sl^{con} rearrangements alter *Mgf* expression through PEV (63) Interestingly, all above mentioned genes are involved in the regulation of transcription Although the association of PEV with transcriptional regulators may suggest a higher sensitivity of transcriptional regulator activity to their chromosomal environment, it is more likely due to the strict stoichiometry that is required for the interactions in which they participate (64) In *Drosophila*, an intracellular concentration-dependent function for the *kruppel* gene has been demonstrated that has repressive activity as a dimer and activating properties as a monomer (65) Therefore, it is conceivable that slight alterations in the transcription levels of these regulators may, unlike many genes where 50% down- or up-regulation often remains unnoticed, disturb a delicate balance in the interactions between transcription factors and their accessory proteins Finally, the variable spreading of X-inactivation into autosomal segments in different cells of individuals with X,a translocations, can also be considered as a mammalian PEV (*chapter 2 3*)

1.3 X Chromosomal Rearrangements: a Special Case

The human X chromosome is the best studied human chromosome due to its unique properties Unlike the autosomes, which are present in two copies, the X chromosome is only present in one functional copy in somatic cells of males and females (see below) Therefore, disorders with an X chromosomal recessive pattern of inheritance are easily recognizable because of their characteristic pattern of transmission

The X chromosome is estimated to comprise 200Mb, which corresponds to approximately 6% of the human genome At present, about 90% of the human X chromosome has already been cloned in overlapping YAC contigs Moreover, more than 150 breakpoints have been identified in individuals with rearranged X chromosomes The regional assignment of these breakpoints has greatly facilitated the integration of the physical and genetic map of the human X chromosome

In males, rearrangements involving the X chromosome may lead to deletion, duplication, disruption, or silencing of the only X chromosomal gene copy which is why they are frequently associated with aberrant phenotypes Also in females, as the result of preferential X-inactivation of the normal or rearranged X chromosome, X chromosomal rearrangements are more frequently associated with disease than autosomal rearrangements Therefore, X chromosomal rearrangements have been extremely valuable tools for the identification of X-linked disease genes (2) X chromosome inactivation, a process exclusively found

in mammals, compensates gene dosage differences between XY males and XX females. The Y chromosome is relatively devoid of genes and its major function is to instruct the undifferentiated gonad to become testis. In contrast, the X chromosome harbors thousands of genes. X-inactivation is a process which in a random manner transcriptionally silences one of the X chromosomes in female somatic cells (66). This inactivation is stable and clonally heritable during subsequent cell divisions. Inactive X chromosomes are easily recognizable as heterochromatic condensed Barr bodies that are attached to the nuclear membrane, and they replicate their DNA late in the S phase (reviews in 67,68). Studies of humans and mice with deletions or translocations involving the X chromosome have shown that the locus determining X-inactivation maps to the proximal long arm of the X chromosome; the X-inactivation centre (XIC) in man (X controlling element (*Xce*) in mice). Four years ago, a gene was cloned that meets all criteria for playing a major role in X-inactivation (68,69). The *XIST* gene (X inactive specific transcript, *Xist* in mice) maps to the XIC locus, is exclusively expressed from the inactive X chromosome (70), and seems to encode a nuclear RNA that is associated with the inactive X chromosome (69,71). X-inactivation can be divided into initiation, spreading and maintenance. XIC is involved in both the initiation and spreading of X-inactivation but not in its maintenance (72). Several genes have been identified that escape X-inactivation in man (reviewed in 73). Some of these genes have been assigned to the X pseudoautosomal region like *MIC2* (74-76) and are expressed from both X chromosomes while others map outside this region and have Y chromosomal homologous genes (*ZFX, RPS4X*) (77,78), or pseudogenes (*STS*) (79,80). Some genes, like *UBE1*, escape inactivation and do not have a homologue on the Y chromosome suggesting that gene dosage is not always critical or that different regulatory mechanisms underlie the expression of these genes in males and females (81,82).

Due to X chromosome inactivation, mammalian cells of both sexes are functionally hemizygous for most X-linked genes. As a result of the randomness of this process during early embryogenesis, and the stable transmission of the X-inactivation status to daughter cells, female tissues and organs are mosaics of cell clones expressing the genes of the maternal or paternal X chromosome, respectively. Females heterozygous for X-linked mutations are mostly asymptomatic or mildly affected as compared with males that carry the mutation. About half of the cells in the relevant tissues and organs express the defect whereas the remainder of the cells express the normal allele. For most of the diseases, this proportion of normal cells is generally high enough to ensure normal functioning of the tissues in the entire organism which is why heterozygotes are mostly unaffected. Occasionally, heterozygous females show the full manifestation of X-linked recessive traits mostly ascribed to 'unfortunate Lyonization'. However, the preferential inactivation of one of the X chromosomes observed in these females may also be caused by the presence of additional X-linked loci involved in the inactivation process. A recently described family in which a grandmaternally inherited X chromosome (and *XIST* allele) is

preferentially inactivated in seven granddaughters while preferentially active in the grandmother supports the presence of these additional loci (83)

Also, other mechanisms may explain skewed X-inactivation observed in females (84-87) Skewed X-inactivation may result from balanced and unbalanced X,a translocations Since initial X-inactivation is generally a random process, cells in which the derivative chromosome carrying *XIST* is inactivated, are apparently at a selective disadvantage Individuals with a preferentially inactivated normal X chromosome mostly have a normal phenotype Sometimes, females carrying a balanced X,a translocation show a specific disease phenotype Disease phenotypes can arise when the normal X chromosome is preferentially inactivated and the translocation disrupts an X chromosomal gene thereby generating functional nullisomy for this locus These cases have been extremely valuable for mapping and cloning of X chromosomal disease genes, as mentioned earlier (2) Interestingly, approximately 20-25% of all females carrying an X,a translocation do not show a complete inactivation of the normal X Typically, as a consequence of aneuploidy, these incomplete X-inactivation patterns may result in a variety of multiple congenital anomalies/mental retardation phenotypes, single gene disorders, and CGSs (88,89) At least partly, variable spreading of X-inactivation from the derivative X chromosome carrying the XIC into the autosomal segment can explain these anomalies The extent of inactivation of autosomal segments by XIC may differ between various cell types, it may be discontinuous, and may even vary within one clone However, the functional X chromosomal disomy resulting from non-inactivation of X chromosomal segments may be equally deleterious since a double dosage of X chromosomal genes is mostly not allowed (89) The presence of active normal X chromosomes in affected skin areas of patients carrying X,a translocations associated with hypomelanosis of Ito not only supports this view but also demonstrates that selection against cells with a double functional dose of X chromosomal genes occurs relatively late during embryonic development (90,91) X,a translocations involving the most distal regions of Xp and Xq are exceptional because they show a more random inactivation Apparently, functional disomies of these outer segments of the X chromosome are, possibly due to their small size and their intrinsic properties (pseudoautosomal regions), compatible with life and are mostly under a mild selective pressure Patients which show a complete inactivation of the translocated X, may have a mutation on their normal X chromosome that is incompatible with life (88,89) Functional Xp disomies are more common than Xq disomies which may be a reflection of the pseudoautosomal region in which a substantial number of genes escape inactivation (89) Therefore, not only the size of the chromosomal segment, but also the nature of the sequences is important for these cellular selection mechanisms Thus, inactivation patterns observed in females with balanced X,a translocations may be the result of balance between gene defects on the normal X on one hand and, an autosomal monosomy and partial disomy X on the other hand

Finally, single gene mutations that cause a growth disadvantage or cell death (e.g. X-linked α-thalassaemia with mental retardation syndrome (92) and X-linked immune deficiencies (93)), or selection for an active mutant X chromosome as in X-linked adrenoleukodystrophy for which the mechanism is unknown (84), may influence the random inactivation pattern of the X chromosome.

1.4 Rearrangements of the Xq21 Band: Association with Blindness, Deafness, and Mental Retardation

The Giemsa-dark staining Xq21 band is relatively devoid of genes which explains why large interstitial deletions of this chromosomal segment can be viable in males. These deletions encompass up to 15 Mb of DNA, which is approximately 8-10% of the human X chromosome (20,94,95). Clinically, these deletions are associated with a CGS consisting of X-linked deafness with a temporal bone defect (DFN3), mental retardation (MR), and choroideremia (CHM), a chorioretinal disorder resulting in peripheral vision loss and eventually in blindness (96-100). Also, deletions have been found in patients with a combination of CHM and DFN3, or CHM and MR depending on the size and position of the deletion (97-99,101,102, *chapters 4 and 5*). The presence of genes for CHM and DFN3 in Xq21 had also been suggested by linkage studies (103-111). The molecular characterization of these deletions has allowed the establishment of the physical order of these disease genes as Xcen-DFN3-MR-CHM-qter, and subsequently the cloning of the genes for CHM (*CHM*) (112,113) and DFN3 (*POU3F4*) (*chapter 6*). Additional clinical features in some patients with deletions in Xq21 are obesity (114), hypogonadism (99), and cleft lip and palate (115). In three families with cleft palate and/or ankyloglossia (CPX), the gene defect has also been linked to Xq21, which supports the presence of a gene involved in palate closure in this segment (116-120).

The gene for MR in Xq21 has not been identified yet. In several families with non-syndromic MR, the underlying gene defect has been mapped to large intervals on the X chromosome that include Xq21.1. Most of these patients are only mildly or moderately affected (121). Moreover, Allan-Herndon-Dudley syndrome (AHDS), which includes severe MR, muscle hypoplasia, spastic paraplegia, and ataxia, has been linked to markers in Xq21 (122,123). However, the severity of MR in these families, as compared to the milder MR in patients carrying interstitial deletions of Xq21.1, and the additional clinical features in AHDS suggests the presence of more than one gene for XLMR in Xq21.

1.5 Aim of this Thesis

In this study, we initially aimed at the cloning of the genes for DFN3 and non-specific MR in Xq21.1. As a prerequisite for the cloning of these genes, we first set out to characterize this chromosomal segment by the generation of YAC contigs

covering the region (*chapters 4 and 5*). This enabled us to improve the physical map of this region, to identify and fine-map several deletion patients, and to further define the critical regions for the MR and DFN3 loci. The actual cloning of the DFN3 gene (*chapter 6*) was eventually aided by the mapping of the murine POU domain transcription factor *Pou3f4* to a segment homologous to the human Xq13.3-q21.1 region (124). The putative presence of transcription regulatory sequences upstream of *POU3F4* and their involvement in DFN3 is further discussed in *chapter 7*.

In order to identify candidate genes for XLMR, we have set out to collect, and map balanced X;a translocations that are associated with MR assuming that disruption of an X chromosomal gene is the cause for the associated phenotype. As a prerequisite for the cloning of these genes, we mapped the X chromosomal breakpoints of MR-associated X;a translocations by fluorescence *in situ* hybridization (*chapter 8*) The apparent colocalization of one of these X chromosomal breakpoints (125) with the deletions of the Xq21.1 band associated with MR, initially prompted us to characterize this breakpoint in detail. The refined localization of this breakpoint in Xq13.1, the molecular analysis and identification of a candidate gene for XLMR is presented in *chapter 9*.

1.6 References

1 McKusick VA. Mendelian Inheritance in Man 10th ed Baltimore John Hopkins University Press, 1992

2 Collins, F S (1995) Positional cloning moves from perditional to traditional *Nature Genet*, **9**, 347-350.

3 Pauling, L , Itano, H A , Singer, S.J , and Wells, I C (1949) Sickle cell anemia a molecular disease. *Science*, **110**, 543.

4 Ingram, V M (1957) Gene mutations in human haemoglobin the chemical difference between normal and cickle cell haemoglobin *Nature*, **180**, 326-328

5 Royer-Pokora, B , Kunkel, L.M , Monaco, A P., Goff, S G , Newburger, P E , Baehner, R L , Cole, F.S , Curnutte, J.T., and Orkin, S H. (1986) Cloning the gene for an inherited human disorder - chronic granulomatous disease - on the basis of its chromosomal localization *Nature*, **322**, 32-38.

6 Riordan, J R , Rommens, J M , Kerem, B -S , Alon, N., Rozmahel, R , Grzelczak, Z , Lok, S , Plavsic, N , Chou, J -L , Drumm, M L., Iannuzzi, M C , Collins, F S , and Tsui, L -C (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA *Science*, **245**, 1066-1073

7 Shenker, A , Laue, L , Kosugi, S , Merendino, J J , Jr , Minegishi, T., and Cutler, G.B , Jr (1993) A constitutively activating mutation of the luteinizing hormone receptor in familial male precocious puberty. *Nature*, **365**, 652-654

8 Kremer, H , Mariman, F , Otten, B J , Moll, G W J , Stoelinga, G B A , Wit, J M , Jansen, M , Drop, S L., Faas, B , Ropers, H -H , and Brunner, H G. (1993) Cosegregation of missense mutations of the luteinizing hormone receptor gene with familial male-limited precocious puberty *Hum Mol Genet*, **2**, 1779-1783

9 Kremer, H., Kraaij, R , Toledo, S P A , Post, M , Fridman, J.B , Hayashida, C.Y , van Reen, M., Milgrom, E., Ropers, H -H., Mariman, E , Themmen, A P N , and Brunner, H G (1995) Male pseudohermaphroditism due to a homozygous missense mutation of the luteinizing hormone receptor gene *Nature Genet*, **9**, 160-164

10 Laue, L., Wu, S -M., Kudo, M , Hsueh, A J W , Cutler, G.B , Jr., Griffin, J E , Wilson, J D , Brain, C., Berry, A C , Grant, D.B , and Chan, W -Y (1995) A nonsense mutation of the human luteinizing hormone receptor gene in Leydig cell hypoplasia *Hum Mol Genet*, **4**, 1429-1433.

11 Cooperative Human Linkage Center (CHLC), Généthon, University of Utah, Yale University, and Centre d'Etude du Polymorphisme Humain (CEPH). (1994) A comprehensive human linkage map with centimorgan density. *Science*, **265**, 2049-2054.

12 Adams, M D., Dubnick, M , Kerlavage, A.R , Moreno, R., Kelley, J.M , Utterback, T.R., Nagle, J W , Fields, C , and Venter, J C. (1992) Sequence identification of 2,375 human brain genes *Nature*, **355**, 632-634

13 Adams, M D., Kerlavage, A R., Fleischmann, R D , Fuldner, R.A , Bult, C J., Lee, N.H., Kirkness, E F , Weinstock, K.G , Gocayne, J D , and et al (1995) Initial assessment of human gene diversity and expression patterns based upon 83 million nucleotides of cDNA sequence *Nature*, **377 supp**, 3-17

14 Burke, D T , Carle, G F , and Olson, M V (1987) Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors *Science*, **236**, 806-812

15 Chumakov, I M , Rigault, P , Le Gall, I , Bellanne-Chantelot, C , Billault, A , Guillou, S , Soularue, P , Guasconi, G , Poullier, E , and et al (1995) A YAC contig map of the human genome *Nature*, **377 supp**, 175-183

16 Leach, F S , Nicolaides, N C , Papadopolous, N , Liu, B , Jen, J , Parsons, R , Peltomaki, P , Sistonen, P , Aaltonen, L A , Nystrom-Lahti, M , Guan, X -Y , Zhang, J , Meltzer, P S , Yu, J -W , Kao, F -T , Chen, D J , Cerosaletti, K M , Fournier, R E K , Todd S , Lewis, T , Leach, R J , Naylor, S N , Weissenbach, J , Mecklin, J -P , Jarvinen, H , Petersen, G M , Hamilton, S R , Green, J , Jass, J , Watson, P , Lynch, H T , Trent, J M , de la Chapelle, A , Kinzler, K W , and Vogelstein B (1993) Mutations of a *mutS* homolog in hereditary nonpolyposis colorectal cancer *Cell* **75** 1215-1225

17 Nicolaides, N C , Papadopolous, N Liu B , Wei, Y -F Carter, K C , Ruben S M , Rosen, C A , Haseltine, W A Fleischmann, R D , Fraser, C M , Adams, M D , Venter, J C , Dunlop, M G , Hamilton, S R , Petersen, G M , de la Chapelle, A , Vogelstein, B , and Kinzler, K W (1994) Mutations of two *PMS* homologues in hereditary nonpolyposis colon cancer *Nature*, **371**, 75-80

18 Service, R F (1994) Stalking the start for colon cancer *Science*, **263**, 1559-1560

19 Warburton, D (1991) De novo balanced chromosome rearrangements and extra marker chromosomes identified at prenatal diagnosis clinical significance and distribution of breakpoints *Am J Hum Genet* , **49**, 995-1013

20 Tommerup, N (1993) Mendelian cytogenetics Chromosome rearrangements associated with mendelian disorders *J Med Genet* , **30**, 713-727

21 Buhler, E M (1983) Unmasking of heterozygosity by inherited balanced translocations Implications for prenatal diagnosis and gene mapping *Ann Genet -Paris*, **26**, 133 137

22 Prockop, D J and Kivirikko, K I (1995) Collagens molecular biology, diseases, and potentials for therapy *Annu Rev Biochem* , **64**, 403-434

23 Park, M (1995) In Scriver, C R , Beaudet, A I , Sly, W S and Valle, D (ed) The metabolic and molecular bases of inherited disease McGraw-Hill,Inc, New York, pp 589 -611

24 Butler, M G (1990) Prader-Willi syndrome current understanding of cause and diagnosis *Am J Med Genet* , **35**, 319-332

25 Pembrey, M , Fennell, S J , Van den Berghe, J , Fitchett, M , Summers, D , Butler, L , Clarke, C , Griffiths, M , Thompson, E , Super, M , and Baraitser, M (1989) The association of Angelman's syndrome with deletions within 15q11-13 *J Med Genet* , **26**, 73-77

26 Norman, A M , Read, A P , Clayton-Smith, J , Andrews, T , and Donnai, D (1992) Recurrent Wiedemann-Beckwith syndrome with inversion of chromosome (11)(p11 2p15 5) *Am J Med Genet* , **42**, 638-641

27 Mannens, M , Hoovers, J M N , Redeker, E , Verjaal, M , Feinberg, A P , Little P , Boavida M , Coad, N , Steenman, M , Bliek, J , Niikawa, N , Tonoki, H , Nakamura, Y , de Boer, E G , Slater, R M , John, R , Cowell, J K , Junien, C , Henry, I , Tommerup, N , Weksberg, R , Pueschel, S M , Leschot, N J , and Westerveld, A (1994) Parental imprinting of human chromosome region 11p15 3-

pter involved in the Beckwith-Wiedemann syndrome and various human neoplasia. *Eur. J Hum Genet*, **2**, 3-23

28 Roberts, R G (1995) In Hall, J C., Dunlap, J.C (ed), Advances in genetics. Academic Press, Inc, San Diego, 33, pp. 177.-231.

29 Rowley, J D (1973) A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and giemsa staining *Nature*, **243**, 290-293.

30 Fitzgerald, P.H. (1976) Evidence that chromosome band 22q12 is concerned with cell proliferation in chronic myeloid leukemia. *Hum Genet.*, **33**, 269-274.

31 de Klein, A., Geurts van Kessel, A , Grosveld, G., Bartram, C R., Hagemeijer, A , Bootsma, D , Spurr, N.K , Heisterkamp, N , Groffen, J., and Stephenson, J.R (1982) A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukaemia. *Nature*, **300**, 765-767

32 Shtivelman, E., Lifshitz, B., Gale, R.P., and Canaani, E (1985) Fused transcript of *abl* and *bcr* genes in chronic myelogenous leukemia. *Nature*, **315**, 550-554.

33 Ballabio, A (1991) Contiguous deletion syndromes. *Curr Opin Genet Dev*, **1**, 25-29

34 Rose, E A , Glaser, T., Jones, C , Smith, C L , Lewis, W H , Call, K M , Minden, M., Champagne, E , Bonetta, L , Yeger, H., and Housman, D.E (1990) Complete physical map of the WAGR region of 11p13 localizes a candidate Wilms' tumor gene *Cell*, **60**, 495-508.

35 Call, K.M , Glaser, T., Ito, C Y., Buckler, A J , Pelletier, J , Haber, D.A , Rose, E A , Kral, A , Yeger, H., Lewis, W H., Jones, C., and Housman, D E (1990) Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus *Cell*, **60**, 509-520.

36 Gessler, M., Poustka, A., Cavenee, W , Neve, R.L., Orkin, S H , and Bruns, G.A.P. (1990) Homozygous deletion in Wilms' tumours of a zinc-finger gene identified by chromosome jumping. *Nature*, **343**, 774-778.

37 Gessler, M., Simola, K.O J., and Bruns, G A.P. (1989) Cloning of breakpoints of a chromosome translocation identifies the AN2 locus. *Science*, **244**, 1575-1578.

38. Ton, C.C T , Hirvonen, H., Miwa, H., Weil, M M., Monaghan, P., Jordan, T., van Heyningen, V., Hastie, N D , Meijers-Heijboer, H , Drechsler, M , Royer-Pokora, B., Collins, F., Swaroop, A., Strong, L C , and Saunders, G F (1991) Positional cloning and characterization of a paired box- and homeobox-containing gene from the aniridia region. *Cell*, **67**, 1059-1074.

39. Jordan, T., Hanson, I., Zaletayev, D , Hodgson, S , Prosser, J , Seawright, A , Hastie, N., and van Heyningen, V. (1992) The human PAX6 gene is mutated in two patients with aniridia. *Nature Genet*, **1**, 328-332.

40 Suter, U and Patel, P.I. (1994) Genetic basis of inherited peripheral neuropathies. *Hum Mutation*, **3**, 95-102.

41 Lupski, J.R., Montes de Oca-Luna, R , Slaugenhaupt, S., Pentao, L., Guzzetta, V , Trask, B J , Saucedo-Cardenas, O , Barker, D F., Killian, J.M., Garcia, C.A , Chakravarti, A., and Patel, P.I. (1991) DNA duplication associated with Charcot-Marie-Tooth disease type 1A *Cell*, **66**, 219-232.

42 Raeymaekers, P , Timmerman, V , Nelis, E , De Jonghe, P., Hoogendijk, J.E., Baas, F , Barker, D F , Martin, J J , de Visser, M , Bolhuis, P A , Van Broeckhoven, C , and the HMSN Collaborative

Research Group (1991) Duplication in chromosome 17p11 2 in Charcot-Marie-Tooth neuropathy type 1a (CMT 1a) *Neuromusc Disord*, **1**, 93-97

43 Pentao, L , Wise, C A , Chinault, A C , Patel, P I , and Lupski, J R (1992) Charcot-Marie-Tooth type 1A duplication appears to arise from recombination at repeat sequences flanking the 1 5 Mb monomer unit *Nature Genet*, **2**, 292-300

44 Chance, P F , Alderson, M K , Leppig, K A , Lensch, M W , Matsunami, N , Smith, B , Swanson, P D , Odelberg, S J , Disteche, C M , and Bird, T D (1993) DNA deletion associated with hereditary neuropathy with liability to pressure palsies *Cell*, **72**, 143-151

45 Lupski, J R , Roth, J R , and Weinstock, G M (1996) Chromosomal duplications in bacteria, fruit flies, and humans *Am J Hum Genet*, **58**, 21-27

46 Valentijn, L J , Baas, F , Wolterman, R A , Hoogendijk, J E , van den Bosch, N , H A , Zorn, I , Gabreels-Festen, A A W M , de Visser, M , and Bolhuis, P A (1992) Identical point mutations of *PMP-22* in *Trembler-J* mouse and Charcot-Marie-Tooth disease type 1A *Nature Genet*, **2**, 288-291

47 Roa, B B , Garcia, C A , Suter, U , Kulpa, D A , Wise, C A , Mueller, J , Welcher, A W , Snipes, G J , Shooter, E M , Patel, P I , and Lupski, J R (1993) Charcot-Marie-Tooth disease type 1A association with a spontaneous point mutation in the *PMP22* gene *New Engl J Med*, **329**, 96-101

48 Muller, H J (1930) Types of visible variations induced by X-rays in Drosophila *J Genet*, **22**, 299-334

49 Spofford, J B (1976) In Ashburner, M , Novitski, E (ed). The genetics and biology of Drosophila Academic Press, New York, 1c, pp 955 -1018

50 Moehrle, A and Paro, R (1994) Spreading the silence epigenetic transcriptional regulation during *Drosophila* development *Dev Genet*, **15**, 478-484

51 Foster, J W , Dominguez-Steglich, M A , Guioli, S , Kwok, C , Weller, P A , Stevanovic, M , Weissenbach, J , Mansour, S , Young, I D , Goodfellow, P N , Brook, J D , and Schafer, A J (1994) Campomelic dysplasia and autosomal sex reversal caused by mutations in an *SRY*-related gene *Nature*, **372**, 525-530

52 Wagner, T , Wirth, J , Meyer, J , Zabel, B , Held, M , Zimmer, J , Pasantes, J , Dagna Bricarelli, F , Keutel, J , Hustert, E , Wolf, U , Tommerup, N , Schempp, W , and Scherer, G (1994) Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the *SRY*-related gene *SOX9 Cell*, **79**, 1111-1120

53 Wirth, J , Wagner, T , Meyer, J , Pfeiffer, R A , Tietze, H U , Schempp, W , and Scherer, G (1996) Translocation breakpoints in three patients with campomelic dysplasia and autosomal sex reversal map more than 130 kb from SOX9 *Hum Genet*, **97**, 186-193

54 Fantes, J , Redeker, B , Breen, M , Boyle, S , Brown, J , Fletcher, J , Jones, S , Bickmore, W , Fukushima, Y , Mannens, M , Danes, S , van Heyningen, V , and Hanson, I (1995) Aniridia-associated cytogenetic rearrangements suggest that a position effect may cause the mutant phenotype *Hum Mol Genet*, **4**, 415-422

55 Busslinger M, Klix N, Pfeffer P, Graninger PG, Kozmik Z (1996) Deregulation of *PAX-5* by translocation of the Em enhancer of the IgH locus adjacent to two alternative *PAX-5* promoters in a diffuse large-cell lymphoma *Proc Natl Acad Sci USA*, **93**, 6129-6134

56 Wilson, C , Bellen, H J , and Gehring, W J (1990) Position effects on eukaryotic gene expression *Ann Rev Cell Biol* , **6**, 679-714

57 Robertson, G , Garrick, D , Wu, W , Kearns, M , Martin, D , and Whitelaw, E (1995) Position-dependent variegation of globin transgene expression in mice *Proc Natl Acad Sci USA* , **92**, 5371-5375

58 Vortkamp, A , Gessler, M , and Grzeschik, K (1991) GLI3 zinc-finger gene interrupted by translocations in Greig syndrome families *Nature* **352**, 539-540

59 Hui, C C and Joyner, A L (1993) A mouse model of Greig cephalopolysyndactyly syndrome the *extra- toes*^{J} mutation contains an intragenic deletion of the *Gli3* gene *Nature Genet* , **3**, 241-246

60 Vortkamp, A , Heid, C , Gessler, M , and Grzeschik, K (1995) Isolation and characterization of a cosmid contig for the GCPS gene region *Hum Genet* , **95**, 82-88

61 Capel, B , Rasberry, C , Dyson, J , Bishop, C E , Simpson, E , Vivian, N , Lovell-Badge, R , Rastan, S , and Cattanach, B M (1993) Deletion of Y chromosome sequences located outside the testis determining region can cause XY female sex reversal *Nature Genet* , **5**, 301-307

62 Laval, S H , Glenister, P H , Rasberry, C , Thornton, C E , Mahadevaiah, S K , Cooke, H J , Burgoyne, P S , and Cattanach, B M (1995) Y chromosome short arm Sxr recombination in XSxr/Y males causes deletion of Rbm and XY female sex reversal *Proc Natl Acad Sci USA* , **92**, 10403-10407

63 Bedell M A , Brannan, C I , Evans E P , Copeland, N G Jenkins, N A , and Donovan, P J (1995) DNA rearrangements located over 100 kb 5' of the Steel (Sl)-coding region in Steel-panda and Steel-contrasted mice deregulate Sl expression and cause female sterility by disrupting ovarian follicle development *Gene Dev* , **9**, 455-470

64 Engelkamp, D and van Heyningen, V (1996) Transription factors in disease *Curr Opin Genet Dev* , **6**, 334-342

65 Sauer, F and Jackle, H (1993) Dimerization and the control of transcription by Kruppel *Nature*, **364**, 454 457

66 Lyon, M F (1962) Sex chromatin and gene action in the mammalian X-chromosome *Am J Hum Genet* , **14**, 135-148

67 Migeon, B R (1994) X-chromosome inactivation molecular mechanisms and genetic consequences *Trends Genet* , **10**, 230-235

68 Rastan, S (1994) X chromosome inactivation and the *Xist* gene *Curr Opin Genet Dev* , **4** 292-297

69 Brockdorff, N , Ashworth, A , Kay, G F , McCabe, V M , Norris, D P , Cooper, P J , Swift, S , and Rastan, S (1992) The product of the mouse *Xist* is a 15 kb inactive X-specific transcript containing no conserved ORF and located in the nucleus *Cell*, **71**, 515-526

70 Brown, C J , Ballabio, A , Rupert, J L , Lafreniere, R G Grompe, M , Tonlorenzi, R , and Willard, H F (1991) A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome *Nature* **349**, 38-44

71 Brown, C J , Hendrich, B D , Rupert J L , Lafreniere, R G , Xing, Y , Lawrence, J , and Willard, H F (1992) The human *XIST* gene analysis of a 17 kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus *Cell*, **71**, 527-542

72 Brown, C J and Willard, H F (1994) The human X-inactivation centre is not required for maintenance of X-chromosome inactivation *Nature*, **368**, 154-156

73 Disteche, C M (1995) Escape from X inactivation in human and mouse *Trends Genet* , **11**, 17-22

74 Goodfellow, P , Banting, G , Sheer D , Ropers, H -H , Caine, A , Ferguson Smith, M A , Povey, S , and Voss, R (1983) Genetic evidence that a Y-linked gene in man is homolgous to a gene on the X chromosome *Nature*, **302**, 346-349

75 Goodfellow, P J , Pym, B , Mohandas, T , and Shapiro, L J (1984) The cell surface antigen locus MIC2X escapes X-inactivation *Am J Hum Genet* , **36**, 777-782

76 Smith, M J , Goodfellow, P J , and Goodfellow, P N (1993) The genomic organisation of the human pseudoautosomal gene *MIC2* and the deletion of a related locus *Hum Mol Genet* , **2**, 417-422

77 Schneider-Gadicke, A , Beer-Romero, P , Brown, L G , Nussbaum, R , and Page, D C (1989) *ZFY* has a gene structure similar to *ZFY*, the putative human sex determinant, and escapes X inactivation *Cell*, **57**, 1247-1258

78 Fisher, E M C , Beer-Romero, P , Brown, L G , Ridley, A , McNeil, J A , Bentley Lawrence, J , Willard, H F , Bieber, F R , and Page, D C (1990) Homologous ribosomal protein genes on the human X and Y chromosomes escape from X inactivation and possible implications for Turner syndrome *Cell*, **63**, 1205-1218

79 Shapiro, L J , Mohandas, T , Weiss, R , and Romeo, G (1979) Non-inactivation of an X-chromosome locus in man *Science*, **204**, 1224-1226

80 Yen, P H , Allen, E , Marsh, B , Mohandas, T , Wang, N , Taggart, R T , and Shapiro, I J (1987) Cloning and expression of steroid sulfatase cDNA and the frequent occurence of deletions in STS deficiency implications for X-Y interchange *Cell*, **49**, 443-454

81 Brown, C J and Willard, H F (1989) Noninactivation of a selectable human X-linked gene that complements a murine temperature-sensitive cell cycle defect *Am J Hum Genet* , **45**, 592-598

82 Brown, C J and Willard, H F (1990) Localization of a gene that escapes inactivation to the X chromosome proximal short arm implications for X inactivation *Am J Hum Genet* , **46**, 273-279

83 Naumova, A K , Plenge, R M , Bird, L M , Leppert, M , Morgan, K , Willard, H F , and Sapienza, C (1996) Heritability of X chromosome-inactivation phenotype in a large family *Am J Hum Genet* , **58**, 1111-1119

84 Migeon, B R , Moser, H W , Moser, A B , Axelman, J , Sillence, D , and Norum, R A (1981) Adrenoleukodystrophy evidence for X linkage, inactivation, and selection favoring the mutant allele in heterozygous cells *Proc Natl Acad Sci USA* , **78**, 5066-5070

85 Fearon, E R , Winkelstein, J A , Civin, C I , Pardoll, D M , and Vogelstein, B (1987) Carrier detection in X-linked agammaglobulinemia by analysis of X-chromosome inactivation *New Engl J Med* , **316**, 427-431

86 Harris, A , Collins, J , Vetrie, D , Cole, C , and Bobrow, M (1992) X inactivation as a mechanism of selection against lethal alleles further investigation of incontinentia pigmenti and X linked lymphoproliferative disease *J Med Genet* , **29**, 608-614

87 Belmont, J.W (1996) Genetic control of X inactivation and processes leading to X-inactivation skewing *Am J Hum Genet* , **58**, 1101-1108

88 Mattei, M G , Mattei, J F., Ayme, S , and Giraud, F. (1982) X-autosome translocations. cytogenetic characteristics and their consequences *Hum Genet* , **61**, 295-309

89 Schmidt, M and Du Sart, D. (1992) Functional disomies of the X chromosome influence the cell selection and hence the X inactivation pattern in females with balanced X-autosome translocations: a review of 122 cases *Am J Med Genet* , **42**, 161-169.

90 Hatchwell, E., Robinson, D., Crolla, J A., Cockwell, A E., Hypomelanosis of ito, X-aurosome translocation, Incontinentia pigmen, and Functional disomy xp. (1996) X inactivation analysis in a female with hypomelanosis of Ito associated with a balanced X;17 translocation evidence for functinal disomy of Xp. *J Med Genet* , **33**, 216-220.

91 Hatchwell, E , Hypomelanosis of ito, X-autosome translocation, and Functional disomy xp (1996) Hypomelanosis of Ito and X,autosome translocations a unifying hypothesis *J Med Genet* , **33**, 177-183

92 Gibbons, R.J., Suthers, G K , Wilkie, A O M , Buckle, V J , and Higgs, D R (1992) X-linked a-thalassemia/mental retardation (ATR-X) syndrome· localization to Xq12-q21 31 by X inactivation and linkage analysis *Am J Hum Genet* , **51**, 1136-1149

93 Belmont, J.W (1995) Insights into lymphocyte development from X-linked immune deficiencies *Trends Genet* , **11**, 112-116

94 Cremers, F.P M., van de Pol, T.J R., Wieringa, B., Hofker, M H., Pearson, P.L , Pfeiffer, R A , Mikkelsen, M , Tabor, A., and Ropers, H. (1988) Molecular analysis of male-viable deletions and duplications allows ordering of 52 DNA probes on proximal Xq *Am J Hum Genet* , **43**, 452-461.

95 Merry, D E., Lesko, J.G , Sosnoski, D.M , Lewis, R A., Lubinsky, M , Trask, B , van den Engh, G , Collins, F S., and Nussbaum, R.L. (1989) Choroideremia and deafness with stapes fixation A contiguous gene deletion syndrome in Xq21 *Am J Hum Genet* , **45**, 530-540

96 Nussbaum, R.L , Lesko, J G., Lewis, R.A., Ledbetter, S A , and Ledbetter, D H (1987) Isolation of anonymous DNA sequences from within a submicroscopic X chromosomal deletion in a patient with choroideremia, deafness, and mental retardation *Proc Natl Acad Sci USA* , **84**, 6521-6525

97 Cremers, F P M , Sankila, E , Brunsmann, F , Jay, M , Jay, B , Wright, A , Pinckers, A J L G , Schwartz, M , van de Pol, T J R , Wieringa, B , de la Chapelle, A., Pawlowitzki, I H , and Ropers, H. (1990) Deletions in patients with classical choroideremia vary in size from 45 kb to several megabases *Am J Hum Genet* , **47**, 622-628

98 Sankila, E , Bruns, G A P., Schwartz, M., Nikoskelainen, E., Niebuhr, E., Hodgson, S V , Wright, A.F., and de la Chapelle, A (1990) DXS26 (HU16) is located in Xq21.1 *Hum Genet* , **85**, 117-120

99 Bach, I , Brunner, H G , Beighton, P , Ruvalcaba, R H A , Reardon, W., Pembrey, M.E., van der Velde-Visser, S D., Bruns, G A P., Cremers, C.W R J , Cremers, F.P.M., and Ropers, H. (1992) Microdeletions in patients with gusher-associated, X-linked mixed deafness (DFN3) *Am J Hum Genet* , **50**, 38-44

100 Bach, I , Robinson, D , Thomas, N , Ropers, H , and Cremers, F.P M (1992) Physical fine mapping of genes underlying X-linked deafness and non fra(X)-X-linked mental retardation at Xq21 *Hum Genet* , **89**, 620-624

101. van Bokhoven, H., van den Hurk, J A J M , Bogerd, L., Philippe, C , Gilgenkrantz, S , de Jong, P , Ropers, H , and Cremers, F P.M. (1994) Cloning and characterization of the human choroideremia gene. *Hum Mol Genet* , **3**, 1041-1046

102 May, M , Collcaux, L , Murgia, A , Aylsworth, A , Nussbaum, R , Fontes, M , and Schwartz, C (1995) Molecular analysis of four males with mental retardation and deletions of Xq21 places the putative MR region in Xq21 1 between DXS233 and CHM *Hum Mol Genet.*, **4**, 1465-1466.

103 Jay, M., Wright, A F., Clayton, J.F., Deans, M , Dempster, M , Bhattacharya, S.S , and Jay, B. (1986) A genetic linkage study of choroideremia *Ophthalmic Paediatr Genet* , 7, 201-204

104 Schwartz, M , Rosenberg, T , Niebuhr, E , Lundsteen, C , Sardemann, H., Andersen, O , Yang, H , and Lamm, L U. (1986) Choroideremia, further evidence for assignment of the locus to Xq13-Xq21 *Hum Genet* , **74**, 449-452

105 Lesko, J G , Lewis, R A., and Nussbaum, R L. (1987) Multipoint linkage analysis of loci in the proximal long arm of the human X chromosome Application to mapping the choroideremia locus *Am J Hum Genet* , **40**, 303-311

106 Sankila, E , de la Chapelle, A , Karna, J , Forsius, H , Frants, R , and Eriksson, A (1987) Choroideremia: Close linkage to DXYS1 and DXYS12 demonstrated by segregation analysis and historical-genealogical evidence. *Clin Genet* , **31**, 315-322

107 Sankila, E., Lehner, T., Eriksson, A.W., Forsius, H , Kärnä, J., Page, D., Ott, J , and de la Chapelle, A (1989) Haplotype and multipoint linkage analysis in Finnish choroideremia families *Hum Genet* , **84**, 66-70

108 Wright, A F., Nussbaum, R L., Bhattacharya, S.S., Jay, M., Lesko, J G., Evans, H.J., and Jay, B. (1990) Linkage studies and deletion screening in choroideremia *J Med Genet* , **27**, 496-498

109. Brunner, H.G., van Bennekom, C A., Lambermon, E M M., Oei, T.L , Cremers, C.W.R J., Wieringa, B., and Ropers, H. (1988) The gene for X-linked progressive mixed deafness with perilymphatic gusher during stapes surgery (DFN3) is linked to PGK. *Hum Genet* , **80**, 337-340.

110. Wallis, C., Ballo, R., Wallis, G , Beighton, P., and Goldblatt, J (1988) X-linked mixed deafness with stapes fixation in a Mauritian kindred linkage to Xq probe pDP34 *Genomics*, **3**, 299-301.

111. Robinson, D , Lamont, M , Curtis, G , Shields, D C , and Phelps, P (1992) A family with X-linked deafness showing linkage to the proximal Xq region of the X chromosome *Hum Genet* , **90**, 316-318

112. Cremers, F P.M , van de Pol, T.J R , van Kerkhoff, E P M , Wieringa, B., and Ropers, H (1990) Cloning of a gene that is rearranged in patients with choroideraemia. *Nature*, **347**, 674-677

113 Merry, D E , Janne, P A , Landers, J E , Lewis, R A , and Nussbaum, R L (1992) Isolation of a candidate gene for choroideremia *Proc Natl Acad Sci USA* , **89**, 2135-2139

114 Ayazi, S (1981) Choroideremia, obesity and congenital deafness *Am J Ophthalmol* , **92**, 63-69

115 Rosenberg, T , Schwartz, M , Niebuhr, E , Yang, H , Sardemann, H , Andersen, O , and Lundsteen, C (1986) Choroideremia in interstitial deletion of the X chromosome *Ophthalmic Paediatr Genet* , **7**, 205-210

116 Moore, G E , Ivens, A , Chambers J , Farrall, M , Williamson, R , Page, D C , Bjornsson, A , Arnason, A , and Jensson, O (1987) Linkage of an X-chromosome cleft palate gene *Nature*, **326**, 91-92

117 Ivens, A , Moore, G E , Chambers, J , Arnason, A , Jensson, O , Bjornsson, A , and Williamson, R (1988) X-linked cleft palate the gene is localized between polymorphic DNA markers DXYS12 and DXS17 *Hum Genet* , **78**, 356-358

118 Gorski, S M , Adams, K J , Birch, P H , Friedman, J M , and Goodfellow, P J (1992) The gene responsible for X-linked cleft palate (CPX) in a British Columbia native kindred is localized between PGK1 and DXYS1 *Am J Hum Genet* , **50**, 1129-1136

119 Stanier, P , Forbes, S A , Arnason, A , Bjornsson, A , Svembjornsdottir, E , Williamson, R , and Moore, G (1993) The localization of a gene causing X-linked cleft palate and ankyloglossia (CPX) in an icelandic kindred is between DXS326 and DXYS1X *Genomics*, **17**, 549-555

120 Gorski, S M , Adams, K J , Birch, P H , Chodirker, B N , Greenberg, C R , and Goodfellow, P J (1994) Linkage analysis of X-linked cleft palate and ankyloglossia in Manitoba Mennonite and British Columbia native kindreds *Hum Genet* , **94**, 141-148

121 Lubs, H A , Chiurazzi, P , Arena, J F , Schwartz, C , Tranebjaerg, L and Neri, G (1996) XLMR genes update 1996 *Am J Med Genet* , **64**, 147-157

122 Schwartz, C E , Ulmer, J , Brown, A , Pancoast, I , Goodman, H O , and Stevenson, R E (1990) Allan-Herndon syndrome II Linkage to DNA markers in Xq21 *Am J Hum Genet* , **47**, 454-458

123 Bialer, M G , Lawrence, L , Stevenson, R E , Silverberg, G , Williams, M K , Arena, JF, Lubs, H A , and Schwartz, C E (1992) Allan-Herndon-Dudley syndrome clinical and linkage studies on a second family *Am J Med Genet* , **43**, 491-497

124 Douville, P J , Atanasoski, S , Tobler, A , Fontana, A , and Schwab, M E (1994) The brain-specific POU-box gene *Brn4* is a sex-linked transcription factor located on the human and mouse X chromosomes *Mamm Genome* , **5**, 180-182

125 Teboul, M , Mujica, P , Chery, M , Leotard, B , and Gilgenkrantz, S (1989) Translocations X-autosomes equilibrees et retard mental Contribution a la cartographie des retards mentaux lies a L'X (a l'exclusion de L'X-FRA) *J Genét Hum* , **37**, 179-195

CHAPTER 2

YEAST ARTIFICIAL CHROMOSOME CLONING OF THE Xq13.3-q21.31 REGION AND FINE MAPPING OF A DELETION ASSOCIATED WITH CHOROIDEREMIA AND NONSPECIFIC MENTAL RETARDATION

van der Maarel, S M , Scholten, I H J M , Maat-Kievit, J A., Huber, I , de Kok, Y J M , de Wijs, I , van de Pol, T J R , van Bokhoven, H , den Dunnen, J T , van Ommen, G J B , Philippe, C , Monaco, A P , Smeets, H J M , Ropers, H , and Cremers, F P M

Eur J Hum Genet , **3**, 207-218 (1995).

S M van der Maarel[a]
I H J M Scholten[a]
J A. Maat-Kievit[b]
I. Huber[a]
Y J M de Kok[a]
I de Wijs[a]
T J R van de Pol[a]
H van Bokhoven[a]
J T den Dunnen[c]
G J B van Ommen[c]
C. Philippe[d]
A.P Monaco[d]
H J M. Smeets[a]
H -H Ropers[a]
F P M Cremers[a]

[a] Department of Human Genetics University Hospital Nijmegen, and
[b] Stichting Klinisch Genetisch Centrum Academisch Ziekenhuis Leiden, and
[c] Department of Human Genetics, Sylvius Laboratories, Leiden, The Netherlands, and
[d] The Wellcome Trust Centre for Human Genetics, Headington, Oxford, UK

Yeast Artificial Chromosome Cloning of the Xq13.3-q21.31 Region and Fine Mapping of a Deletion Associated with Choroideremia and Nonspecific Mental Retardation

Key Words
Mental retardation · Choroideremia · DFN3 · Deletions · Yeast artificial chromosome · X chromosome · Xq21

Abstract
Microscopically detectable deletions and X;autosome translocations have previously facilitated the construction of a high-resolution interval map of the Xq21 region. Here, we have generated three yeast artificial chromosome contigs spanning approximately 7 megabases of the Xq13.3-q21.31 region. In addition, a novel deletion associated with choroideremia and mental retardation was identified and mapped in detail. The proximal deletion endpoint was positioned between the loci DXS995 and DXS232, which enabled us to confirm the critical region for a locus involved in mental retardation. The distal deletion endpoint is situated in the Xq21.33 band, which allowed us to refine the order of several markers in this region.

Introduction

In the Giemsa-dark-staining Xq21 band, numerous large male-viable deletions have been described which encompass up to 15 megabases (Mb) of DNA, or 8–10% of the estimated size of the human X chromosome [1, 2]. These deletions often give rise to contiguous gene syndromes including choroideremia (CHM), X-linked deafness (DFN3) and

nonspecific mental retardation (MR) [3–7]. Sizeable deletions have also been found in patients with nonsyndromic CHM or DFN3 [4–6, 8, 9].

Employing positional cloning strategies, the genes underlying CHM and DFN3 have been isolated. Analysis of patients with Xq21 deletions and syndromic and nonsyndromic forms of MR has enabled us to map the gene for X-linked MR to a region between CHM and DFN3 [4, 7, 10–13].

As a prerequisite for the isolation of this and other genes in Xq21, we have set out to generate a ladder of overlapping yeast artificial chromosomes (YACs) spanning the entire Xq21 region To this end, we first employed numerous X;autosome translocations and deletions to subdivide the Xq21 band into 24 different intervals [8, 14; Philippe, unpubl. results]. Moreover, we have previously described a 850-kb YAC and cosmid contig encompassing the DFN3 locus and a 350-kb YAC encompassing the CHM gene [9, 12, 13]. Here, we report on three YAC contigs spanning approximately 7 Mb of the Xq13.3-q21.31 region and on the localization of a novel deletion associated with CHM and MR.

Materials and Methods

Patients

All patients except patient AP have been described elsewhere and are listed in table 1.

YAC Clones

YAC clones were initially identified by PCR amplification of YAC DNA pools or by hybridization of probes to filters containing gridded YAC DNA YACs were isolated from previously described CEPH [15]

Table 1. Patients included in this study

Patient	Clinical features	Cytogenetic abnormality	References
3 5	CHM	46,Y,del(X)(Xq21.1)	4, 28, 46
MBU	CHM, seizures MR[a]	46,Y,del(X)(q21.1-q22)	27, 47
LGL2905	CHM	46,Y,del(X)(Xq21 1-q21 31)	4, 5
AP	CHM, MR	46,Y,del(X)(Xq21.1-q21.33)	this manuscript
D20	CHM, MR, seizures, DFN3	46,Y,del(X)(Xq21.1-q21.33)	7, 48
TD	DFN3, hypogonadism	46,Y,del(X)(Xq21 1)	6, 36
1/10	DFN3	46,Y,del(X)(Xq21.1)	6, 49
XL62	CHM, MR, DFN3	46,Y,del(X)(Xq21.1-q21.33)	3, 27
C56N	amenorrhoea	46,X,t(X,5)(q21,q11)	14
NP	CHM, MR, cleft lip and palate	46,Y,del(X)(Xq21.1-q21.33)	1, 30, 50
RvD	MR, hearing impairment	46,Y,del(X)(Xq13.3-q21.33)	1

[a] The mild mental handicap does not cosegregate with the deletion in this family [47]

and ICRF YAC libraries [16] All relevant markers used in this study are listed in table 2

Positive YAC clones were plated on NZYM agar [17], and three yeast clones each were picked and cultured as described elsewhere [18] Yeast cells were embedded in low-melting agarose, incubated for 2 h in the presence of zymolyase (Seikagu) and incubated overnight with pronase (Boehringer Mannheim) Yeast chromosomes were separated using pulsed-field gel electrophoresis (PFGE) and blotted to a GeneScreen Plus membrane (NEN Dupont) YAC blots were hybridized with ^{32}P-labelled total human DNA to estimate the size of the human insert Subsequently, the identity of the YACs was checked with the marker originally used for their isolation YACs were further characterized by screening of adjacent markers and through the generation of left- and right-end fragments by ligation-mediated PCR (LM-PCR, see below)

In addition, we screened the CEPH YAC data base with new DNA markers from this region and for YAC clones which overlap with previously mapped YACs

Southern Blot Analysis

Hybridization of ^{32}P-labelled DNA fragments to nylon filters containing DNA from patients or from YAC clones was essentially done as described elsewhere [9]

LM-PCR and Sequence Analysis

The LM-PCR method was employed essentially as described by Kere et al [19] Yeast DNA was digested with *Alu*I, *Eco*RV, *Rsa*I or *Pvu*II prior to ligation of adaptors LM-PCR end fragment products of the YAC clones were separated on an agarose gel and purified with the Qiaquick gel extraction kit (Quiagen) Purified DNA was sequenced using fluorescent dideoxynucleotides on an Applied Biosystems 373A DNA sequencer Sequencing reactions were done with a Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions The oligonucleotides of end fragment 753e10EV/L (ATTTATTCTTCCAGGCCCATA and CGGAATTCTGAAAAAGAGATC) were designed (Isogen Bioscience, The Netherlands) to generate a product of 94 bp For end fragment 5045H5, the oligonucleotides TGGATAAAGTAAAAAGCACACAAG and TGGTAGTTGTCTCATAGCTCTTG amplify a product of 260 bp Finally, a 152-bp PCR product (A0822EV/L) can be amplified from the left end of YAC A0822 with the oligonucleotides CCTAATTTGGGAAGAACATATC and AATTTAAGAGAGAAATTATTCATATT

Table 2. Markers used in this study

Locus	Name	References
PGK1	pHPGK-7e	51
DXS566	HX60	52
DXS986	AFM116Xg1	53, 54
DXS1197	AFM072za5	54
DXS346	RX86	55
DXS1225	AFM311v9	54
DXS738	MIT-E114	56
DXS72	pX65H7	57
DXS169	pX104F	57
DXS26	pHU16	5
DXS995	AFM207Zg5	53, 54
POU3F4	pBRN4 1	13
DXS232	pJL68	3
DXS121	p784	58
DXS233	pJL8	3
DXS326	pQST38M1	55
DXS1167	D3	55
DXS1209	AFM273zd5	54
ZNF6	CMPX1	23
DXS165	p1bD5	59, 60
CHM	p864	12
DXS540	pZ11	61
DXS1002	AFM249vh5	53, 54
DXS95	pXG7c	59, 62
DXS1168	XKL10	55
DXS364	RX272	55
DXS349	pRXG8H3	55
DXS262	K20-40	63
DXS110	p722	58, 59
DXS472	pXG8b	1, 62
DXS3	p19 2	59, 64
DXS1170	B8	55
DXS112	p753	58, 59
DXS990	AFM136yc7	53, 54
DXS458	Mfd79	65

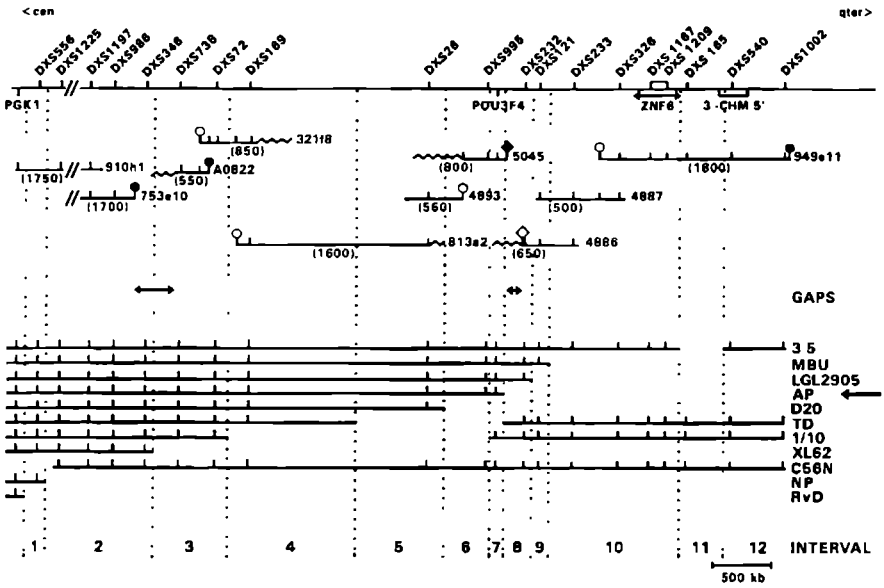


Fig. 1. Physical map of the Xq13.3-q21.3 region. On the upper horizontal bar, the previously and newly mapped DNA loci are indicated. The YACs are represented by solid bars for X chromosomal sequences and wavy bars for autosomal sequences. Vertical bars indicate the presence of the markers on the YACs and in the DNA of patients with deletions. Open circles are right-end fragments while solid circles indicate left-end fragments of the YACs. Solid and open diamonds represent the outermost X-chromosomal cosmid clones derived from their respective YACs used for direct-visual-hybridization analysis. The identification numbers of the YACs are depicted adjacent to the YACs, while their estimated sizes are depicted underneath in parentheses. The gaps between the three YAC contigs are indicated with double arrowheads. The deletions found in several patients used in this study are depicted at the bottom of the figure. The solid bar represents the chromosomal segments present in the patients. The molecular characterization of all patients has been reported elsewhere except for patient AP (indicated with an arrow), who suffers from MR and CHM. The mapping results of the YAC end fragments are only indicated for overlapping YACs. For purposes of clarity, the region between POU3F4 and DXS233 is not true to scale but expanded twofold. At the bottom, the intervals from this region as defined by Philippe et al. [14, unpubl. data] are depicted.

Results

Construction of a YAC Contig

Several markers from the Xq21 region were employed to screen the ICRF and CEPH YAC libraries [15, 16]. All YAC clones were extensively screened for the presence of X-linked markers and for the absence of autosomal sequences. In this way, a YAC map was generated consisting of three nonoverlapping contigs encompassing the Xq13.3-q21.31 region. From these contigs, YAC 4893 and

YAC 5045 have been published elsewhere [9] To establish novel sequence-tagged sites (STSs) and to check the integrity of the YACs, we employed the LM-PCR technique [19] LM-PCR products were hybridized to adjacent YACs and to DNAs of a large number of patients with deletions in Xq21 to localize and orient the YACs (fig 1) End fragments mapping to the Xq21 region were sequenced, and oligonucleotides were designed for PCR amplification to generate new STSs

The middle and most distal YAC contigs span approximately 3 and 2 5 Mb, respectively Although the size of the most proximal contig has not been determined precisely, we estimate that together, the resulting YAC contigs encompass a chromosomal segment of approximately 7 Mb, extending from PGK1 proximally to DXS1002 distally with a gap between DXS986 and DXS738 and between end fragment 5045H5 and the DXS232 locus The most proximal contig is linked to a previously published YAC contig spanning the PGK1 locus [20, 21] The most distal contig overlaps a previously reported YAC contig in the Xq21 3 region [22]

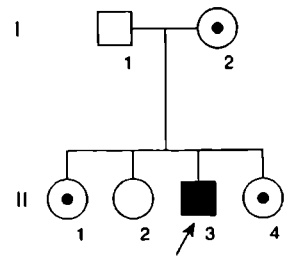


Fig. 2. Pedigree of kindred with CHM and MR Patient AP (II-3) was ascertained via his sister (II-4) who was prenatally counselled Two of his sisters (II-1 and II-4) and his mother (I-2) are carriers of the deletion AP, his father (I-1) and one of his sisters (II-4) also carry a balanced translocation t(10,15)(q26 3,q24 3) II 1 and II-2 have not been tested for chromosomal abnormalities

Identification and Fine Mapping of a Deletion Associated with CHM and MR

Patient AP is a 40-year-old mentally retarded man with CHM Ophthalmologic findings suggest that two of his sisters (II-1 and II-4) and his mother (I-2) are carriers of CHM Apart from a microdeletion in the Xq21 band (see below) patient AP carries a balanced translocation 46,XY,t(10,15) (q26 3,q24 3), which is also present in his mentally normal sister (II-4) and father, and apparently is not associated with a clinical phenotype (fig 2) II-1 and II-2 have not been investigated for the presence of the translocation

Patient AP was investigated for the presence of a deletion by PCR amplification of several exons from the CHM gene All exons tested were absent, which prompted us to investigate the extent of the deletion in greater detail On the proximal side, the markers DXS26 and DXS995 were found to be present in patient AP, while DXS232 and DXS121 were absent Also, the POU3F4 gene, previously shown to be involved in DFN3, appeared to be present in this patient [13] These findings located the proximal breakpoint of patient AP between the previously defined intervals 7 and 8 of the Xq21 band (fig 1) [14, Philippe, unpubl data] Subsequently, several cosmids from a previously published 850-kb cosmid contig [9] were hybridized to precisely map the proximal endpoint of this deletion The breakpoint is located in cosmid 5045H3, which detects a novel restriction fragment carrying the deletion

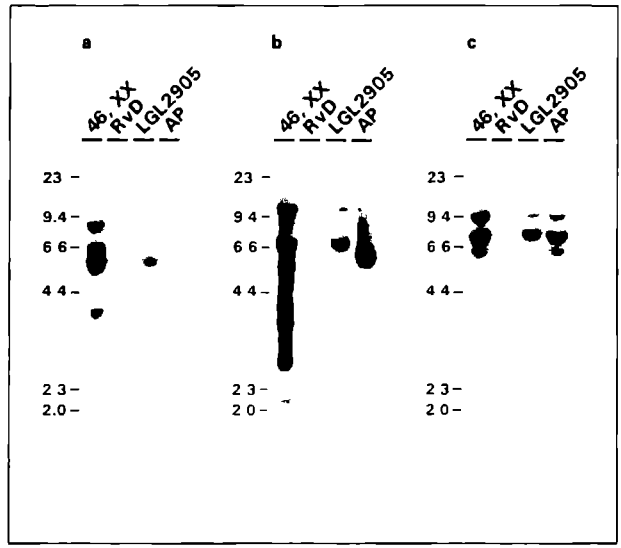


Fig. 3. Mapping of the proximal deletion breakpoint in patient AP Hybridization of the cosmids 5045E7 (**a**), 5045H3 (**b**) and 5045A6 (**c**) to a Southern blot of *Eco*RI-digested DNA of patients with deletions and a female control In patient AP, cosmid 5045H3 detects a novel restriction fragment indicative of the proximal breakpoint of the deletion.

junction (fig 3) A cosmid located proximal to this breakpoint (cosmid 5045A6) is entirely present in patient AP, while the first cosmid (5045E7) distal to cosmid 5045H3 is completely absent. These results confirm the initial localization of the breakpoint in cosmid 5045H3 (fig. 3). It is noteworthy that the distal breakpoint of another deletion, detected previously in a patient with DFN3 (TD) [9], is also located in cosmid 5045H3, approximately 10 kb from the proximal deletion breakpoint in patient AP (data not shown).

Several markers from the Xq21.2-q21.31 region, i.e. DXS1002, DXS95, DXS262, DXS110 and DXS472, appeared to be absent in patient AP, thus the distal breakpoint of the deletion in patient AP could be assigned to the Xq21.32-q21.33 segment. To localize this breakpoint more precisely, several markers from a previously defined interval (interval 20) [14; Philippe, unpubl. data] were employed PCR analysis revealed that DXS3 is absent, while DXS1170 and DXS990 are present in the DNA of AP. Conventional Southern blot analysis showed that the DXS112 locus is also located proximal to this deletion breakpoint (data not shown) Therefore, the distal breakpoint of the AP deletion subdivides this interval [Philippe, unpubl. data] in two distinct intervals: 20A and 20B. The loci DXS3 and DXS112 are located in interval 20A, whereas interval 20B harbors DXS1170 and DXS990.

Mapping of New Markers with Respect to Known Markers

These data and the previously established deletion interval map [14, Philippe et al., unpubl data] have enabled us to define the physical order for several markers. Four markers have been mapped to interval 2 based on their presence in patient XL62 and in C56N, a human-rodent cell hybrid containing the Xq21.1-qter segment [Philippe et al., unpubl data]. DXS1225 is present on YAC 910h1 and absent from YAC 752e10. Therefore, this marker is located proximal to DXS1197 and DXS986 which are both present on YAC 753e10. Since DXS1197 is present on both above-mentioned YACs, the order must be Xcen-DXS1225-DXS1197-DXS986-Xqter. DXS346 also maps to interval 2 and is absent in both YACs. Since the left-end fragment of YAC 753e10 (753e10EV/L) is also located in this interval (data not shown), DXS346 can be placed distal to this end fragment.

DXS738 is located in interval 3, based on its presence in patient 1/10 and absence from patient XL62 (fig. 1) [Philippe et al., unpubl data]. DXS738 is absent from both YAC 753e10 and 321f8, which positions it proximal to DXS72. Since the end fragment A0822EV/L of YAC A0822 is also present in YAC 321f8 while DXS72 is absent from YAC A0822, the end fragment is positioned proximal from DXS72 in interval 3. By combining these results with previous mapping data [14, Philippe et al., unpubl data] we propose the following order: Xcen-PGK1-DXS566-DXS1225-DXS1197-DXS896-753e10EV/L-DXS346 - DXS738 - A0822EV/L - DXS72 - DXS169-Xqter.

A third relevant end fragment, 5045H5, maps distal to POU3F4 and proximal to DXS232. This end fragment is located ouside the deletions found in patients TD and LGL2905, which assigns this marker to interval 8. 753e10EV/L, A0822EV/L and 5045H5 were sequenced and oligonucleotides were designed for PCR amplification. In this way, three new STSs could be generated (see Materials and Methods).

More distally, DXS1167 bould be placed between DXS326 and DXS165 because of its localization on YAC 949e11 and its absence from YAC 4887. This marker has been mapped previously to interval 10 [14, Philippe et al., unpubl data] (fig. 1). DXS1209 and ZNF6, a gene containing a zinc finger motif, which has been mapped to Xq21 [23] could be localized in the same way. DXS95, DXS349, DXS364 and DXS1168 could be placed distal to DXS1002 on the basis of their absence from YAC 949e11. A comprehensive overview of the Xq21 region, including the YAC contigs, all above-mentioned markers and the relevant deletions is given in figure 1.

Discussion

In this study we have established three YAC contigs encompassing the Xq13.3-q21.31 region except for a gap between DXS986 and DXS738, and a gap between POU3F4 and DXS232. To estimate the size of the latter gap, cosmids from the proximal segment of YAC 4886 and the most distal cosmid of YAC 5045 were employed as probes for direct-visual-hybridization analyses [24] (fig. 1). These experiments revealed that the size of the gap does not exceed 100 kb [Merkx and van der Maarel, unpubl data]. We are currently screening for cosmids from the ICRF Cosmid Reference Library [25] and Chromosome X Cosmid Library LL0XNC01 'U' to fill this gap. Despite extensive screening of the ICRF and CEPH YAC libraries [15, 16], we have not yet been able to fill the remaining two gaps. The generation of the end clone 753e10EV/L will hopefully lead to

the identification of new YACs between DXS986 and DXS738

We confirmed the localization of 19 DNA markers and 4 genes from the Xq13 3-Xq21 31 region and were able to map them in the correct order In addition, three STS clones were generated and positioned in the contigs

We assume that the DXS566 locus maps to an internal deletion in YAC 910h1, because in this YAC, the respective primer pair has failed to yield a PCR product We cannot rule out the possibility that DXS346 also maps to this deletion interval If so, DXS346 should be located between DXS566 and DXS1225

Many of the microscopically detectable deletions in the Xq21 region are associated with DFN3, MR and CHM [2, 6, 7, 26, 27] The identification of submicroscopic deletions associated with either CHM [28] or DFN3 [6, 9] has enabled the subsequent cloning of the relevant genes [10–13] Characterization of these deletions has revealed that the physical order of these disease genes is Xcen-DFN3-MR-CHM-qter Some patients with deletions in Xq21 show clinical features which are not observed in others and which do not seem to fit the contiguous gene syndrome model The most striking additional features are obesity [29], hypogonadism [6] and cleft lip and palate [30] The presence of a gene in Xq21 involved in palate closure is also suggested by linkage studies in three families with X-linked cleft palate and/or ankyloglossia (CPX) The critical region for CPX was demarcated proximally by DXS1002 and distally by DXYS1 [Stanier, pers commun , 31–35] In view of the large number of deletions known in Xq21, it is striking that thus far no small deletions have been found with nonspecific mental retardation or with a combination of MR with either DFN3 or CHM

Here, we are the first to report on a deletion associated with MR and CHM The deletion encompasses the CHM gene and extends into the chromosomal region between the DFN3 and CHM genes confirming the initial localization of a MR locus in Xq21 1 Since the deletion in AP encompasses a large segment of Xq21, one could speculate that the deletion disrupts a MR gene in Xq21 33, distal to the CHM gene There are two arguments against this hypothesis First, the MR gene would be located distal to several deletions associated with a complex phenotype including MR, e g XL45, SD, and DM Second, two patients with nonsyndromic CHM have been described [8], in whom deletions extend into or beyond interval 20 (patient LUN3 and LUN1) Together, these data strongly suggest that a locus for XLMR is situated in the chromosomal region defined by intervals 8, 9 and 10 At the proximal side, this region is demarcated by the deletions found in DFN3 patients II/7, 1/10, and TD [9] The proximal deletion breakpoint in AP does not significantly narrow down the localization of the MR locus, since it is located only 10 kb from the distal deletion breakpoint in TD At the distal side, deletions associated with CHM in patients C759, 3 5, 25 6, MS, and 7 6 [8] commence between ZNF6 and DXS165 and extend telomerically The deletion in patient LGL2905 who has CHM but no MR, restricts the critical region for MR to interval 8

It is remarkable that apart from DFN3, patient TD shows hypogonadism and mild MR Five affected family members are not mentally retarded but show marked antisocial and immature behavior and possibly mild learning deficits [36] Since the deletion in patient TD is close to the MR locus [7], it could affect the proper transcription of the MR gene This might explain the complex clinical findings in TD and his family

So far, we found no evidence for microdeletions in the critical region for MR (interval

8) in 40 patients with XLMR [van der Maarel, unpubl. results], whereas in patients with CHM or DFN3, deletions were detected in approximately 25% of cases [8, 9; de Kok and Cremers, unpubl. results]. It is very likely that the main reason for this discrepancy is the genetic heterogeneity of XLMR [37]. Indeed, the Xq21.1 locus may only be involved in a small proportion of cases with XLMR. Thus far, several syndromic forms of MR, like Allan-Herndon-Dudley syndrome, Juberg-Marsidi, and α-thalassemia/MR (ATR-X) have been mapped to Xq13-q21 [38–43], but recent linkage studies of our group in families with nonspecific XLMR point to a clustering in the Xp11 region [44]. Recently, mutations in a putative global transcriptional regulator (XH2) have been shown to be causative for ATR-X [45]

Definite proof for the existence of a gene for MR in Xq21.1 can only be obtained by cloning of candidate genes from the relevant region and identification of mutations in syndromic or nonsyndromic cases of XLMR. These studies will be greatly facilitated by the Xq13.3-q21 31 YAC contigs reported here.

Acknowledgements

The authors thank Mrs. S D van der Velde-Visser and E. Boender-van Rossum for expert technical assistance in cell culturing and W.K.C van Loon and Y. Ishikawa-Brush for screening the YAC libraries in the context of the YAC Screening Center grant GENE-CT-0093-0088 of the European Community The chromosome-specific gene library LL0XNC01 used in this work was constructed at the Biology and Biotechnology Research Program, Lawrence Livermore National Laboratory, Livermore, CA 94550, USA, under the auspices of the National Gene Library Project sponsored by the U S Department of Energy We thank Drs M Ross, G Zehetner and H Lehrach for access to the ICRF cosmid library filters. The research of FPMC has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences. This study was supported by the Netherlands Organization for Scientific Research (NWO grants 901-04-125 and 900-716-801), the Dutch Praeventiefonds (grant 28-2447) and the European Community (grant GENE-CT93-0022)

References

1 Cremers FPM, van de Pol TJR Wieringa B, Hofker MH, Pearson PL, Pfeiffer RA, Mikkelsen M, Tabor A, Ropers HH Molecular analysis of male-viable deletions and duplications allows ordering of 52 DNA probes on proximal Xq Am J Hum Genet 1988,43 452–461

2 Merry DE, Lesko JG, Sosnoski DM Lewis RA, Lubinsky M, Trask B, van den Engh G, Collins FS, Nussbaum RL Choroideremia and deafness with stapes fixation A contiguous gene deletion syndrome in Xq21 Am J Hum Genet 1989,45 530–540

3 Nussbaum RL, Lesko JG Lewis RA, Ledbetter SA, Ledbetter DH Isolation of anonymous DNA sequences from within a submicroscopic X chromosomal deletion in a patient with choroideremia, deafness, and mental retardation Proc Natl Acad Sci USA 1987,84 6521–6525

4 Cremers FPM Sankila EM Brunsmann F, Jay M, Jay B, Wright A, Pinckers AJLG, Schwartz M, van de Pol TJR, Wieringa B, de la Chapelle A, Pawlowitzki IH, Ropers HH Deletions in patients with classical choroideremia vary in size from 45 kb to several megabases Am J Hum Genet 1990,47 622–628

5 Sankila EM, Bruns GAP, Schwartz M, Nikoskelainen E, Niebuhr E, Hodgson SV, Wright AF, de la Chapelle A DXS26 (HU16) is located in Xq21 1 Hum Genet 1990,85 117–120

6 Bach I, Brunner HG, Beighton P, Ruvalcaba RHA. Reardon W, Pembrey ME, van der Velde-Visser SD Bruns GAP, Cremers CWRJ, Cremers FPM Ropers HH Microdeletions in patients with gusher-associated, X-linked mixed deafness (DFN3) Am J Hum Genet 1992,50 38–44

7 Bach I, Robinson D, Thomas N, Ropers HH Cremers FPM Physical fine mapping of genes underlying X-linked deafness and non fra(X)-X-linked mental retardation at Xq21 Hum Genet 1992,89 620–624

8 van Bokhoven H, Schwartz M, Andreasson S van den Hurk JAJM Bogerd L, Jay M, Ruther K, Jay B, Pawlowitzki IH, Sankila EM, Wright A, Ropers HH, Rosenberg T, Cremers FPM Mutation spectrum in the CHM gene of Danish and Swedish choroideremia patients Hum Mol Genet 1994 3 1047–1051

9 Huber I, Bitner-Glindzicz M, de Kok YJM, van der Maarel SM, Ishikawa-Brush Y, Monaco AP, Robinson D, Malcolm S, Pembrey ME, Brunner HG, Cremers FPM, Ropers HH X-linked mixed deafness (DFN3) Cloning and characterization of the critical region allows the identification of novel microdeletions Hum Mol Genet 1994,3 1151–1154

10 Cremers FPM van de Pol TJR, van Kerkhoff EPM, Wieringa B, Ropers HH Cloning of a gene that is rearranged in patients with choroideraemia Nature 1990 347 674–677

11 Merry DE, Janne PA, Landers JE, Lewis RA, Nussbaum RL Isolation of a candidate gene for choroideremia Proc Natl Acad Sci USA 1992 89 2135–2139

12 van Bokhoven H, van den Hurk JAJM, Bogerd L, Philippe C, Gilgenkrantz S, de Jong P, Ropers HH, Cremers FPM Cloning and characterization of the human choroideremia gene Hum Mol Genet 1994,3 1041–1046

13 de Kok YJM van der Maarel SM, Bitner-Glindzicz M Huber I Monaco AP Malcolm S, Pembrey ME, Ropers HH, Cremers FPM Association between X-linked mixed deafness and mutations in the POU domain gene POU3F4 Science 1995, 267 685–688

14 Philippe C, Cremers FPM, Chery M, Bach I, Abbadi N, Ropers HH, Gilgenkrantz S Physical mapping of DNA markers in the q13-q22 region of the human X chromosome Genomics 1993,17 147–152

15 Albertsen HM, Abderrahim H, Cann HM, Dausset J, Le Paslier D, Cohen D Construction and characterization of a yeast artificial chromosome library containing seven haploid human genome equivalents Proc Natl Acad Sci USA 1990,87 4256–4260

16 Larin Z, Monaco AP, Lehrach H Yeast artificial chromosome libraries containing large inserts from mouse and human DNA Proc Natl Acad Sci USA 1991 88 4123–4127

17 Maniatis T, Fritsch EF, Sambrook J Molecular Cloning A Laboratory Manual Cold Spring Harbor, Cold Spring Harbor Laboratory, 1982

18 Green ED, Olson MV Systematic screening of yeast artificial-chromosome libraries by use of the polymerase chain reaction Proc Natl Acad Sci USA 1990,87 1213–1217

19 Kere J, Nagaraja R, Mumm S, Ciccodicola A, D'Urso M, Schlessinger D Mapping human chromosomes by walking with sequence-tagged sites from end fragments of yeast artificial chromosome inserts Genomics 1992,14 241–248

20 Gecz J, Villard L, Lossi AM, Millasseau P, Djabali M, Fontes M Physical and transcriptional mapping of DXS56-PGK1 1 Mb region Identification of three new transcripts Hum Mol Genet 1993,2 1389–1396

21 Villard L, Gecz J, Collcaux L, Lossi AM, Chelly J, Ishikawa-Brush Y, Monaco AP, Fontes M Construction of a YAC contig spanning the Xq13 3 subband Genomics 1995, 26 115–122

22 Stanier P Forbes S, Richardson M, Brennan L, Cole CG, Bentley DR, Moore GE Physical mapping of the X-linked cleft palate locus Cytogenet Cell Genet 1994,67.351

23 Lloyd SL, Sargent CA, Chalmers J, Lim E, Habeebu SSM, Affara NA An X-linked zinc finger gene mapping to Xq21 1–q21 3 closely related to ZFX and ZFY Possible origins from a common ancestral gene Nucleic Acids Res 1991,19 4835–4841

24 Parra I, Windle B High resolution visual mapping of stretched DNA by fluorescent hybridization Nat Genet 1993,5 17–21

25 Zehetner G, Lehrach H The reference library system-sharing biological material and experimental data Nature 1994,367 489–491

26 Cremers FPM, van de Pol TJR, Wieringa B, Collins FS, Sankila EM, Siu VM, Flintoff WF, Brunsmann F, Blonden LAJ, Ropers HH Chromosomal jumping from the DXS165 locus allows molecular characterization of four microdeletions and a de novo chromosome X/13 translocation associated with choroideremia Prox Natl Acad Sci USA 1989,86 7510–7514

27 Cremers FPM, van de Pol TJR, Diergaarde PJ, Wieringa B, Nussbaum RL, Schwartz M, Ropers HH Physical fine mapping of the choroideremia locus using Xq21 deletions associated with complex syndromes. Genomics 1989,4 41–46

28 Cremers FPM, Brunsmann F, van de Pol TJR, Pawlowitzki IH, Paulsen K, Wieringa B, Ropers HH. Deletion of the DXS165 locus in patients with classical choroideremia Clin Genet 1987,32 421–423

29 Ayazi S Choroideremia, obesity and congenital deafness Am J Ophthalmol 1981,92 63–69

30 Rosenberg T, Schwartz M, Niebuhr E, Yang HM, Sardemann H, Andersen O, Lundsteen C Choroideremia in interstitial deletion of the X chromosome Ophthalmic Paediatr Genet 1986,7 205–210

31 Moore GE, Ivens A, Chambers J, Farrall M, Williamson R, Page DC, Bjornsson A, Arnason A, Jensson O Linkage of an X-chromosome cleft palate gene Nature 1987,326 91–92

32 Ivens A Moore GE Chambers J, Arnason A Jensson O Bjornsson A, Williamson R X-linked cleft palate The gene is localized between polymorphic DNA markers DXYS12 and DXS17 Hum Genet 1988 78 356–358
33 Gorski SM, Adams KJ, Birch PH, Friedman JM Goodfellow PJ The gene responsible for X-linked cleft palate (CPX) in a British Columbia native kindred is localized between PGK1 and DXYS1 Am J Hum Genet 1992,50 1129–1136
34 Stanier P, Forbes SA, Arnason A, Bjornsson A, Sveinbjornsdottir E, Williamson R, Moore G The localization of a gene causing X-linked cleft palate and ankyloglossia (CPX) in an Icelandic kindred is between DXS326 and DXYS1X Genomics 1993,17 549–555
35 Gorski SM, Adams KJ, Birch PH, Chodirker BN, Greenberg CR, Goodfellow PJ Linkage analysis of X-linked cleft palate and ankyloglossia in Manitoba Mennonite and British Columbia native kindreds Hum Genet 1994,94 141–148
36 Myhre SA, Ruvalcaba RHA, Kelley VC Congenital deafness and hypogonadism A new X-linked recessive disorder Clin Genet 1982;22 299–307
37 Neri G, Chiurazzi P, Arena JF, Lubs A XLMR genes Update 1994 Am J Med Genet 1994,51 542–549
38 Schwartz CE, Ulmer J, Brown A, Pancoast I, Goodman HO, Stevenson RE Allan-Herndon syndrome II Linkage to DNA markers in Xq21 Am J Hum Genet 1990,47 454–458.
39 Schwartz CE, Martin J, Ouzts L, Arena JF Lubs HA, Stevenson RE Allan-Herndon-Dudley syndrome Linkage analysis in a third family and refinement of the localization in Xq21 Cytogenet Cell Genet 1994, 67 351
40 Saugier-Veber P, Abadie V, Moncla A, Mathieu M, Piussan C, Turleau C, Mattei JF, Munnich A, Lyonnet S The Juberg-Marsidi syndrome maps to the proximal long arm of the X chromosome (Xq12-q21) Am J Hum Genet 1993,52 1040–1045
41 Gibbons RJ, Suthers GK, Wilkie AOM Buckle VJ, Higgs DR X-linked α-thalassemia/mental retardation (ATR-X) syndrome Localization to Xq12-q21 31 by X inactivation and linkage analysis Am J Hum Genet 1992,51 1136–1149
42 Houdayer C, Toutain A, Ronce N, Lefort G Sarda P, Taib J, Briault S Lambert JC, Moraine C X-linked α-thalassemia/mental retardation syndrome Linkage analysis in a new family further supports localization in proximal Xq Ann Genet 1993, 36 194–199
43 Lefort G, Taib J, Toutain A, Houdayer C Moraine C, Humeau C, Sarda P X-linked α-thalassemia/mental retardation (ATR-X) syndrome Ann Genet 1993,36 200–205
44 Ropers HH, van der Maarel S, Knoers N, Kremer H, Smits A, Hamel B, Cremers F, Gilgenkrantz S, Philippe C, Monaco T, Ishikawa-Brush Y, Smeets D, Mariman E 'Unspecific' X-linked mental retardation Clinical, genetic and molecular studies Am J Hum Genet 1994, 55 A49
45 Gibbons RJ, Picketts DJ, Villard L, Higgs DR Mutations in a putative global transcriptional regulator cause X-linked mental retardation with α-thalassemia (ATR-X syndrome) Cell 1995,80 837–845
46 Hammerstein W, Bohm T Fluoreszenzangiographische Verlaufsbeobachtungen bei Chorioideremie Stuttgart, Enke, 1985, pp 300–303
47 Hodgson SV, Robertson ME, Fear CN, Goodship J, Malcolm S, Jay B, Bobrow M, Pembrey ME Prenatal diagnosis of X-linked choroideremia with mental retardation, associated with a cytologically detectable X-chromosome deletion Hum Genet 1987,75 286–290
48 Wells S, Mould S, Robins D, Robinson D, Jacobs P Molecular and cytogenetic analysis of a familial microdeletion of Xq J Med Genet 1991,28 163–166
49 Wallis C, Ballo R, Wallis G, Beighton P, Goldblatt J X-linked mixed deafness with stapes fixation in a Mauritian kindred Linkage to Xq probe pDP34 Genomics 1988,3 299–301
50 Tabor A, Andersen O, Lundsteen C, Niebuhr E, Sardemann H Interstitial deletion in the 'critical region' of the long arm of the X chromosome in a mentally retarded boy and his normal mother. Hum Genet 1983, 64 196–199
51 Willard HF, Goss SJ, Holmes MT, Munroe DL Regional localization of the phosphoglycerate kinase gene and pseudogene on the human X chromosome and assignment of a related DNA sequence to chromosome 19 Hum Genet 1985,71 138–143
52 Porteous MEM, Curtis A, Lindsay S, Williams O, Goudie D, Kamakari S, Bhattacharya SS The gene for Aarskog syndrome is located between DSX255 and DXS566 (Xp11 2-Xq13) Genomics 1992,14 298–301
53 Weissenbach J, Gyapay G, Dib C, Vignal A, Morissette J, Millasseau P, Vaysseix G, Lathrop M A second-generation linkage map of the human genome Nature 1992,359 794–801.
54 Gyapay G, Morissette J, Vignal A, Dib C, Fizames C, Millasseau P, Marc S, Bernardi G, Lathrop M, Weissenbach J: The 1993–94 Genethon human genetic linkage map Nat Genet 1994;7 246–339
55 Barker DF, Fain PR Definition and mapping of STSs at STR and RFLP loci in Xp11-Xq22 Genomics 1993, 18 712–716
56 Hudson TJ, Engelstein M, Lee MK, Ho EC, Rubenfield MJ, Adams CP, Housman DE, Dracopoli NC Isolation and chromosomal assignment of 100 highly informative human simple sequence repeat polymorphisms Genomics 1992;13 622–629
57 Riddell DC, Wang HS, Beckett J, Chan A, Holden JJA, Mulligan LM, Phillips MA, Simpson NE, Wrogemann K, Hamerton JL, White BN Regional localization of 18 human X-linked DNA sequences Cytogenet Cell Genet 1986,42 123–128.

58 Hofker MH, Bergen AAB, Skraastad MI, Carpenter NJ, Veenema H, Connor JM, Bakker E van Ommen GJB, Pearson PL Efficient isolation of X chromosome-specific single-copy probes from a cosmid library of a human X/hamster hybrid-cell line Mapping of new probes close to the locus for X-linked mental retardation Am J Hum Genet 1987,40 312–328

59 Goodfellow PN, Davies KE, Ropers HH Report of the committee on the genetic constitution of the X and Y chromosomes Human Gene Mapping 8 Eighth International Workshop on Human Gene Mapping Cytogenet Cell Genet 1985,40 296–352

60 Paulsen K, Forrest S, Scherer G, Ropers HH, Davies K Regional localisation of X chromosome short arm probes Hum Genet 1986,74 155–159

61 van de Pol TJR, Cremers FPM, Brohet RM Wieringa B Ropers HH Derivation of clones from the choroideremia locus by preparative field inversion gel electrophoresis Nucleic Acids Res 1990,18 725–731

62 Davatelis G, Siniscalco M, Szabo P Toward a more complete linkage map of the human X-chromosome Cytogenet Cell Genet 1985,40 611

63 Dietz-Band JN, Turco AE, Willard HF, Vincent A, Skolnick MH, Barker DF Isolation, characterization, and physical localization of 33 human X-chromosome RFLP markers Cytogenet Cell Genet 1990,54 137–141

64 Aldridge J, Kunkel L, Bruns G, Tantravahi U, Lalande M, Brewster T, Moreau E, Wilson M, Bromley W, Roderick T, Latt S A strategy to reveal high-frequency RFLPs along the human X chromosome Am J Hum Genet 1984,36 546–564

65 Weber JL, Kwitek AE, May PE, Polymeropoulos MH, Ledbetter S Dinucleotide repeat polymorphisms at the DXS453, DXS454 and DXS458 loci Nucleic Acids Res 1990,18 4037

CHAPTER 3

X-LINKED MIXED DEAFNESS (DFN3): CLONING AND CHARACTERIZATION OF THE CRITICAL REGION ALLOWS THE IDENTIFICATION OF NOVEL MICRODELETIONS

Huber, I , Bitner-Glindzicz, M , de Kok, Y J M , van der Maarel, S M , Ishikawa-Brush, Y , Monaco, A P , Robinson, D , Malcolm, S , Pembrey, M E , Brunner, H G , Cremers, F P M , and Ropers, H

Hum Mol Genet , **3**, 1151-1154 (1994)

X-linked mixed deafness (DFN3): cloning and characterization of the critical region allows the identification of novel microdeletions

Irene Huber, Maria Bitner-Glindzicz[1], Yvette J M.de Kok, Silvère M.van der Maarel, Yumiko Ishikawa-Brush[2], Anthony P.Monaco[2], David Robinson[3], Susan Malcolm[1], Marcus E.Pembrey[1], Han G.Brunner, Frans P.M.Cremers* and Hans-Hilger Ropers

Department of Human Genetics University Hospital Nijmegen PO Box 9101 6500 HB Nijmegen The Netherlands [1]Institute of Child Health University of London 30 Guilford Street London WC1N 1EH [2]University of Oxford Institute of Molecular Medicine John Radcliffe Hospital Headington Oxford OX3 9DU and Wessex Regional Genetics Laboratory Salisbury District Hospital Oldstock Salisbury SP2 8BJ UK

Received March 30 1994 Revised and Accepted May 6 1994

We have found that the microsatellite marker AFM207zg5 (DXS995) maps to all previously described deletions which are associated with X-linked mixed deafness (DFN3) with or without choroideremia and mental retardation. Employing this marker and pHU16 (DXS26) we have identified two partially overlapping yeast artificial chromosome clones which were used to construct a complete 850 kb cosmid contig. Cosmids from this contig have been tested by Southern blot analysis on DNA from 16 unrelated males with X-linked deafness. Two novel microdeletions were detected in patients which exhibit the characteristic DFN3 phenotype. Both deletions are completely contained within one of the known DFN3-deletions, but one of them does not overlap with two previously described deletions in patients with contiguous gene syndromes consisting of DFN3, choroideremia, and mental retardation. Assuming that only a single gene is involved, this suggests that the DFN3 gene spans a chromosomal region of at least 400 kb.

INTRODUCTION

X-linked mixed deafness with perilymphatic gusher at surgery in males (DFN3, MIM 304400) is characterized by a severe hearing loss involving all frequencies, that may be progressive About 50% of female heterozygotes show mild to moderate hearing impairment Audiological studies indicate a basic sensorineural hearing loss, with the variable conductive element postulated to reflect outward perilymphatic pressure splinting the stapes footplate in the oval window (1) On opening of the stapes footplate during surgery, an abnormal amount of perilymph is encountered Computerized tomography (CT) studies have demonstrated an abnormal dilatation of the internal acoustic canal (IAC) as well as an insufficient structural separation between the IAC and the basal coil of the cochlea (2–4)

Linkage studies in families with X-linked deafness have assigned the underlying gene to the Xq13–q21 region (4–7) This localization is supported by the phenotype of patients with cytogenetically visible deletions of Xq21 region, which includes choroideremia (CHM), mental retardation (MR) and DFN3 (8–12) Molecular characterization of Xq21 deletions has enabled us and others to refine the assignment of the DFN3 gene to a small segment of Xq21 1 (13–15) Subsequent examination of patients with classic DFN3 revealed the existence of two submicroscopic deletions encompassing DXS26 (cases TD and 1/10, Figure 1) (16)

We now have employed yeast artificial chromosome (YAC) and cosmid contig cloning to characterize a 850 kb chromosomal segment encompassing DXS26 and DXS995 This has enabled us to fine map several classic and syndromic DFN3 deletions and to identify two novel microdeletions Some of the deletions are non-overlapping, which suggests that we have cloned a sizeable part of the DFN3 gene in cosmid clones

RESULTS

Identification of YACs from the DFN3 region and construction of a cosmid contig

Physical mapping of several recently isolated X-chromosomal markers using a deletion panel described by Philippe *et al* (17) revealed that the microsatellite marker AFM207zg5 (DXS995) does not only map to large deletions of the Xq21 band which are associated with X-linked deafness, CHM, and MR, but also to two previously described microdeletions (TD and 1/10) in patients with non-syndromic DFN3 (Philippe *et al.* in preparation)

By screening a human YAC library (18) with the probes AFM207zg5 and pHU16, we were able to isolate two YAC clones, nos 5045 and 4893, respectively Physical mapping of YAC endclones generated by the ligation-mediated polymerase chain reaction (LM-PCR) procedure (19), indicated that YAC 5045 is chimaeric containing an autosomal fragment at its proximal side (Figure 1) High molecular weight DNA from both YACs was used to construct cosmid libraries consisting of approximately 2500 independent clones for each YAC clone Cosmids containing human inserts were identified and gridded on to several nylon membranes Cosmid contigs were established by colony hybridization or Southern blot analysis of *Eco*RI-digested cosmid DNAs with pHU16, YAC endclones, and iterative hybridization of cosmid inserts Two *Eco*RI restriction maps were constructed spanning a total of approximately 800

*To whom correspondence should be addressed

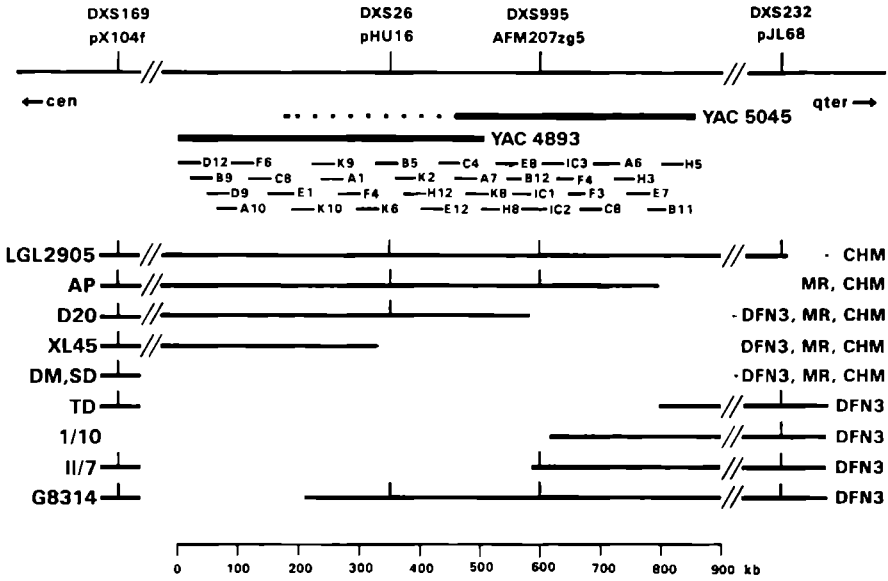


Figure 1 Physical map of the DFN3 region The X chromosomal parts of the YACs are given in the upper part as bars The position of the cosmids subcloned from the YACs or identified in the ICRF X chromosome cosmid library by cosmid walking (IC1 – IC3) are depicted below the YACs Deleted segments are depicted by stippled lines The clinical features of the patients are given on the right side

kb YAC 4893 was completely represented in the contig whereas both the distal end of YAC 5045 as well as the chromosomal region spanning DXS995 were found to be absent

To close the gap in the vicinity of DXS995, cosmids 5045F4 and 5045B12 were used to perform cosmid walking employing the ICRF Cosmid Reference Library (20) Three rounds of cosmid walking resulted in the identification of three new cosmids (IC1 – IC3), which close the cosmid gap (Figure 1)

Fine mapping of deletions associated with complex syndromes and identification of novel microdeletions in DFN3 patients

*Not*I/*Bss*HII inserts of the cosmid clones indicated in Figure 1 were hybridized to *Eco*RI or *Taq*I digested DNAs from syndromic and classic DFN3 patients as well as to *Eco*RI digested DNA from a patient showing choroideremia and mental retardation The latter patient, AP, has a deletion which encompasses exons 2 through 15 of the CHM gene as well as the more proximally located DNA markers DXS165 DXS233 DXS121, and DXS232 (van der Maarel *et al* in preparation) The proximal deletion endpoint is situated in cosmid 5045B11 approximately 30 kb from the distal end of the cosmid contig Detailed analysis of the deletions found in the syndromic' DFN3 patients D20 and XL45 positioned their respective breakpoints just proximal to DXS995 and DXS26, respectively (Figure 1) The proximal deletion breakpoints in other patients with syndromic deafness (DM and SD) mapped proximal to the cosmid contig and therefore do not provide additional mapping information

Southern blot analysis of DNA from 16 unrelated patients with non-syndromic X linked deafness using all cosmids from the contig resulted in the identification of two novel microdeletions, II 7 and G8314 as shown in Figure 2 This figure is a typical example of using *Not*I/*Bss*HII cosmid inserts in a competitive DNA hybridization assay The deletion in patient 1/10 encompasses the DNA loci DXS995, DXS26, and DXS169 The deletions in patients II 7 and G8314 merely span DXS26 and thus are entirely contained within the microdeletion seen in patient 1/10 (Figure 1) Interestingly the G8314 deletion does not overlap the deletions of patients D20 and XL45 The distance between the proximal deletion endpoint in D20 and the distal deletion endpoint in G8314 is approximately 400 kb

DISCUSSION

We have established a cosmid contig spanning the critical region for DFN3 and have identified two novel, highly informative deletions associated with X-linked deafness Taken together, we have now found four microdeletions (TD, 1/10 II/7, and G8314) among 16 patients with X linked deafness In all four patients with microdeletions the bony changes indicative of DFN3 were observed For two patients suffering from X linked deafness choroideremia and mental retardation, D20 and XL45 the deletions were shown not to overlap with the deletion in patient G8314 In patient D20, the structural changes of the inner ear indicative of DFN3 has been demonstrated by CT (21) Although patient XL45 was reported to have sensorineural deafness (22)

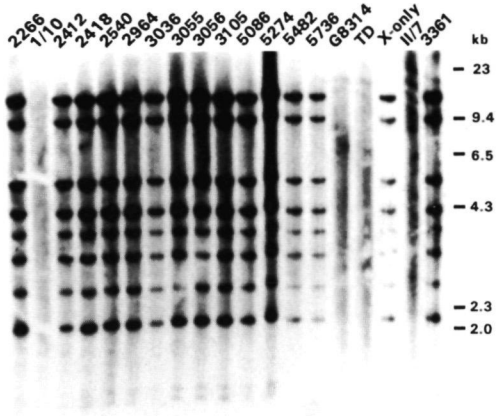


Figure 2. Southern blot analysis of *Eco*RI digested genomic DNAs from patients with X-linked deafness employing cosmid 4893E1. Size markers are given to the right. X-only represents cell line 578, a hamster cell line containing one human X chromosome.

it is possible that the DFN3 gene is involved in this case too. Glasscock (23) hypothesized that a very rapid progression of the sensorineural component of DFN3 can mask the conductive element. Since in a younger brother of XL45 mixed deafness was diagnosed (13), it is tempting to speculate that deafness in both brothers is caused by a (partial) deletion of the DFN3 gene. Linkage studies suggest that X-linked sensorineural deafness is genetically heterogeneous (7), but this has not been demonstrated for the mixed type of X-linked deafness. Assuming that only one gene is involved in DFN3, our results indicate that the DFN3 gene spans a chromosomal segment of at least 400 kb containing both the DXS995 and DXS26 loci. However, it is possible that there are additional rearrangements in the X-chromosome of some of the patients with deletions. Within the limits of resolution of conventional Southern blotting, this has been excluded in the cosmid contig region for patients TD, G8314 and II/7, but not for patients 1/10, D20, and XL45. If the rearrangements in the DFN3 patients presented here are not complex, the DFN3 gene might well be the second largest gene known to date, after the Duchenne muscular dystrophy (DMD) gene which spans approximately 2.3 Mb (24–26). The DMD and DFN3 genes are located in Xp21 and Xq21, respectively, the most prominent Giemsa dark-staining regions of the X-chromosome. Another gene from Xq21, the choroideremia gene, also spans a sizeable (>150 kb) chromosomal segment (27). Only two of 15 deletions associated with classic CHM are intragenic; the others extend both proximal and distal of the gene spanning a chromosomal segment of approximately 15 Mb (14,27). The classic DFN3 deletions described here encompass chromosomal segments of a minimum of 250 kb (patient G8314) to several megabases (patient 1/10). Together, these findings support the idea that the Xq21 band contains a relatively small number of large genes. If so, this has important implications for cloning strategies aiming at the identification of sequences of the DFN3 gene proper.

We did not find smaller-sized deletions among our X-linked deafness patients, which might narrow down the search for some of the protein coding sequences of the DFN3 gene. In the cosmid contig presented here, no CpG island indicative of the presence of the 5′ end of the DFN3 gene was found. It is, however, possible that a sizeable portion of the gene is located proximal to YAC 4893. To investigate this chromosomal segment, we have identified a YAC clone spanning DXS26 and DXS169, which is currently under investigation.

Assuming the existence of widely spaced small exons, as for example in the DMD and CHM genes, it might be difficult to find exons of the DFN3 gene by analysing cosmid clones for evolutionary conserved sequences. Alternative methods as for example exon trapping and cDNA enrichment protocols are underway, which should soon enable us to identify DFN3 protein coding sequences.

MATERIAL AND METHODS

Patients

All patients except AP, II/7, and G8314 have been extensively described elsewhere (15,16; and references therein). Patient AP suffers from CHM and MR (van der Maarel *et al.*, in preparation). Patients II/7 and G8314 show the classical features of DFN3 including the typical CT scan changes of the inner ear (4; M.Bitner-Glindzicz, unpublished data).

YAC clone screening and construction of YAC cosmid contigs

YAC 4893 (ICRFy901E1023) was isolated by hybridization with pHU16 from primary library filters of the human ICRF human YAC library (18). YAC 5045 (ICRFy900B1210) was identified by a PCR screening protocol from pooled DNAs from the same library. Yeast cell culturing and DNA isolation was performed as described elsewhere (28). YAC endclones were generated by the ligation mediated polymerase chain reaction (LM-PCR) protocol essentially as described by Kere *et al.* (19). Restriction enzyme digestion of yeast DNA prior to ligation of adaptors was done with *Alu*I, *Eco*RV, *Rsa*I, or *Pvu*II. The procedure used for constructing cosmid libraries in the SuperCos 1 vector (Stratagene) has been described elsewhere (29).

Cosmid walking

DNA was isolated from cosmids 5045F4 and 5045B12 using a Qiagen midi-prep kit and digested using either *Eco*RI or *Pst*I according to manufacturer's instructions. Fragments were Southern blotted and hybridized with radiolabelled human placental DNA (Sigma), washed in 0.1×SSC, 0.1% (w/v) SDS for 30 min at 65°C and exposed to X-ray film for 3–24 h at −70°C. A band showing no signal was selected as a probe for the next part of the walk, excised from a 0.8% agarose gel and spun through glass wool at 13 000 r.p.m. for 10 min. Probes were preannealed with human competitor DNA.

ICRF gridded X chromosome cosmid filters were hybridized as described by Nizetic *et al.* (30) and positive colonies selected. Digests of DNA from positive colonies were compared with those of flanking cosmids, blotted and probed with the band which had originally been used to select them from the gridded filter, to ensure that they were true positive colonies. The whole process of probing with human placental DNA and band selection was repeated at the next stage of the walk. Three such rounds of hybridization and walking were needed to bridge the gap in the cosmid contig around DXS995 and resulted in the identification of the following clones: IC1 (ICRFc104P0517), IC2 (ICRFc104L0131), IC3 (ICRFc104B1939).

Southern blot analysis and DNA markers

Methods employed for the isolation of high-molecular-weight DNA, restriction-endonuclease digestion, as well as separation and blotting of DNA fragments have been described elsewhere (15). Hybridization of the cosmid inserts in the presence of excess human competitor DNA was done as described by Blonden *et al.* (31). Hybridized filters were washed at 65°C with 40 mM Na_2HPO_4 (pH 7.2)/0.5% (w/v) SDS for 3×5 min and 1×30 min. Autoradiography took 4–16 h at −70°C using two intensifying screens.

The markers pJL68 (DXS232), pHU16 (DXS26), pX104f (DXS169), and AFM207zg5 (DXS995) have been described elsewhere (9,32–34).

ACKNOWLEDGEMENTS

The authors are indebted to Drs P Beighton C Cremers J Gouldsmith O Michel P Thorpe, R Ruvalcaba W Reardon and K Shaver-Arnos for providing blood from patients with X linked mixed deafness We thank S D van der Velde-Visser and E Boender van Rossum for technical assistance in cell culturing We thank Drs M Ross, G Zehetner and H Lehrach for access to ICRF YAC and cosmid library filters PCR pools and the Reference Library Database The research of F P M C has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences M B-G is an MRC Clinical Training Fellow M E P is funded by Mothercare This work was supported by the Netherlands Organization for Scientific Research (NWO) and by the Imperial Cancer Research Fund

ABBREVIATIONS

CHM choroideremia CT computerized tomography DFN3, X linked mixed deafness, MR, mental retardation, YAC yeast artificial chromosome

REFERENCES

1 Reardon W Bellman S, Phelps P, Pembrey, M and Luxon, L M (1993) *Acta Otolaryngol* **113** 706–714
2 Michel, O, Breunsbach, J and Matthias, R (1991) *HNO* **39** 486–490
3 Phelps, P D, Reardon, W, Pembrey, M E, Bellman, S and Luxom, L (1991) *Neuroradiology* **33** 326–330
4 Robinson D Lamont, M, Curtis G, Shields, D C and Phelps, P D (1992) *Hum Genet* **90** 316–318
5 Brunner, H G, van Bennekom, C A, Lambermon E M M, Oei T L, Cremers, C W R J Wieringa, B and Ropers H H (1988) *Hum Genet* **80** 337–340
6 Wallis, C, Ballo, R, Wallis G, Beighton, P and Goldblatt, J (1988) *Genomics* **3** 299–301
7 Reardon, W, Middleton-Price H R Sandkuijl, L, Phelps, P, Bellman, S, Luxon, L, Pembrey, M E and Malcolm, S (1991) *Genomics* **11** 885–894
8 Rosenberg, T, Schwartz, M, Niebuhr, E Yang, H M, Sardemann, H, Andersen, O and Lundsteen C (1986) *Ophthalmic Paediatr Genet* **7** 205–210
9 Nussbaum R L, Lesko, J G, Lewis, R A, Ledbetter, S A and Ledbetter, D H (1987) *Proc Natl Acad Sci USA* **84** 6521–6525
10 Cremers, F P M, van de Pol, T J R, Wieringa, B, Hofker M H, Pearson, P L, Pfeiffer R A, Mikkelsen, M, Tabor, A and Ropers, H -H (1988) *Am. J Hum Genet* **43** 452–461
11 Cremers, F P M, van de Pol, T J R, Diergaarde, P J, Wieringa, B, Nussbaum, R L, Schwartz M and Ropers, H -H (1989) *Genomics* **4** 41–46.
12 Reardon, W, Roberts, S, Phelps, P D, Thomas, N S, Beck, L, Issac, R and Hughes, H E (1992) *Am J Med Genet* **44** 513–517
13 Merry, D E, Lesko J G Sosnoski D M Lewis, R A, Lubinsky R M, Trask B, van den Engh, G Collins F S and Nussbaum, R L (1989) *Am J Hum Genet* **45** 530–540
14 Cremers, F P M, Sankila, E -M, Brunsmann, F, Jay, M, Jay, B, Wright, A, Pinckers, A J L G, Schwartz, M, van de Pol, T J R Wieringa, B, de la Chapelle, A, Pawlowitzki, I H and Ropers, H H (1990) *Am J Hum Genet* **47** 622–628
15 Bach, I, Robinson, D Thomas, N Ropers, H H and Cremers, F P M (1992) *Hum Genet* **89** 620–624
16 Bach, I, Brunner, H G, Beighton, P, Ruvalcaba, R H A, Reardon, W, Pembrey, M E, van der Velde Visser, S D, Bruns, G A P, Cremers, C W R J, Cremers, F P M and Ropers, H -H (1992) *Am J Hum Genet* **51** 38–44
17 Philippe, C, Cremers F P M Chery, M., Bach, I, Abbadi, N, Ropers, H H and Gilgenkrantz S (1993) *Genomics* **17** 147–152
18 Larin, Z, Monaco, A P and Lehrach, H (1991) *Proc Natl Acad Sci USA* **88** 4123–4127
19 Kere, J, Nagaraja, R, Mumm, S, Ciccodicola, A, D'Urso, M and Schlessinger D (1992) *Genomics* **14** 241–248
20 Zehetner, G and Lehrach H (1994) *Nature* **367** 489–491
21 Wells, S, Mould, S Robins, D, Robinson, D and Jacobs, P (1991) *J Med Genet* **28** 163–166
22 Ayazi, S (1981) *Am J Ophthalmol* **92** 63–69
23 Glasscock, M E (1973) *Arch Otolaryngol* **98** 82–91
24 Kenwrick S Patterson, M, Speer A Fischbeck K and Davies K (1987) *Cell* **48** 351–357
25 Burmeister M, Monaco, A P Gillard, E F, van Ommen, G J B Affara, N A, Ferguson-Smith, M A, Kunkel, L M and Lehrach H (1988) *Genomics* **2** 189–202
26 Van Ommen, G J B Bertelson, C, Ginjaar, H B den Dunnen, J T Bakker, E Chelly J, Matton, M, van Essen, A J, Bartley, J, Kunkel L M and Pearson, P L (1987) *Genomics* **1** 329–336
27 van Bokhoven, H, van den Hurk, J A J M, Bogerd, L Philippe, C, Gilgenkrantz, S, de Jong, P., Ropers, H -H and Cremers, F P M (1994) *Hum Mol Genet* **3** 1041–1046
28 Green, E D and Olson, M V (1990) *Proc Natl Acad Sci USA* **87** 1213–1217
29 Berger, W, Meindl, A, van de Pol, T J R, Cremers, F P M Ropers H H, Doerner, C, Monaco, A P, Bergen, A A B, Lebo, R Warburg M Zergollern, L, Lorenz, B Gal A, Bleeker-Wagemakers, E M and Mettinger T (1992) *Hum Mol Genet* **1** 199–203
30 Nizetic, D, Zehetner, G, Monaco A P, Gellen, L, Young, B D and Lehrach, H (1991) *Proc Natl Acad Sci USA* **88** 3233–3237
31 Blonden L A J, den Dunnen, J T van Paassen, H M B, Wapenaar M C Grootscholten, P M, Ginjaar H B, Bakker, E, Pearson, P L and van Ommen, G J B (1989) *Nucleic Acids Res* **17** 5611–5621
32 Sankila E M, Bruns, G A P, Schwartz, M, Nikoskelainen, E Niebuhr, E Hodgson S V, Wright, A F and de la Chapelle, A (1990) *Hum Genet* **85** 117–120
33 Ruddell, D C, Wang, H S, Becker, J, Chan, A, Holden, J J A, Mulligan, L M, Phillips, M A, Simpson, N E, Wrogemann, K, Hamerton, J L and White, B N (1986) *Cytogenet Cell Genet* **42** 123–128
34 Weissenbach J, Gyapay, G, Dib, C, Vignal, A, Morissette, J, Millasseau, P, Vaysseix G and Lathrop, M (1992) *Nature* **359** 794–801

CHAPTER 4

ASSOCIATION BETWEEN X-LINKED MIXED DEAFNESS AND MUTATIONS IN THE POU DOMAIN GENE POU3F4

de Kok, Y J M , van der Maarel, S M , Bitner-Glindzicz, M , Huber, I , Monaco, A P , Malcolm, S , Pembrey, M E , Ropers, H , and Cremers, F P M

Science , **267**, 685-688 (1995)

Association Between X-Linked Mixed Deafness and Mutations in the POU Domain Gene *POU3F4*

Yvette J. M. de Kok,* Silvère M. van der Maarel,* Maria Bitner-Glindzicz, Irene Huber, Anthony P. Monaco, Susan Malcolm, Marcus E. Pembrey, Hans-Hilger Ropers, Frans P. M. Cremers†

Deafness with fixation of the stapes (DFN3) is the most frequent X-linked form of hearing impairment. The underlying gene has been localized to a 500-kilobase segment of the Xq21 band. Here, it is reported that a candidate gene for this disorder, *Brain 4* (*POU3F4*), which encodes a transcription factor with a POU domain, maps to the same interval. In five unrelated patients with DFN3 but not in 50 normal controls, small mutations were found that result in truncation of the predicted protein or in nonconservative amino acid substitutions. These findings indicate that *POU3F4* mutations are a molecular cause of DFN3.

Severe, inherited childhood deafness occurs in about 1 out of 1000 births and presents a serious worldwide public health problem (*1*). In 70% of these cases, deafness is not associated with other clinically recognizable features (*2*). To date, genes for nonsyndromic sensorineural deafness have been mapped to five different autosomes (*3*), but none of these has been isolated yet. The most frequent cause of X-linked hearing impairment, X-linked mixed deafness (DFN3) (McKusick catalog number 304400), is characterized by a conductive hearing loss that results from stapes fixation and progressive sensorineural deafness (*4, 5*). However, a profound sensorineural deafness sometimes masks the conductive element (*6*). Computerized tomography (CT) studies in people with DFN3 demonstrated an abnormal dilatation of the internal acoustic canal (IAC) as well as an abnormally wide communication between the IAC and the inner ear compartment (*7*). As a result, there is an increased perilymphatic pressure that is thought to underlie the observed "gusher" during the opening of the stapes footplate. The gene underlying DFN3 has been mapped to Xq21 by linkage analysis (*8, 9*) and through molecular characterization of large and submicroscopic deletions (*10, 11*). Yeast artificial chromosome (YAC) clones that span the critical region were isolated, and an 850 kb cosmid contig was constructed (*12*). This enabled us to identify and characterize two additional microdeletions, as well as a 150 kb duplication in patients with DFN3, and to assign the gene underlying DFN3 to a 500 kb interval of Xq21.1 (*12, 13*) (Fig. 1B).

Recently, the gene *Brain 4* (*Pou3f4*), which codes for a transcription factor, was mapped between the proteolipid protein locus *Plp* and the *DXMit6* marker near the phosphoglycerate kinase 1 (*Pgk1*) gene on the murine X chromosome (*14*). The chromosomal region between *Pgk1* and *Plp* is evolutionarily conserved between humans and mice, which suggests that the human *POU3F4* gene is located in the Xq13-q22

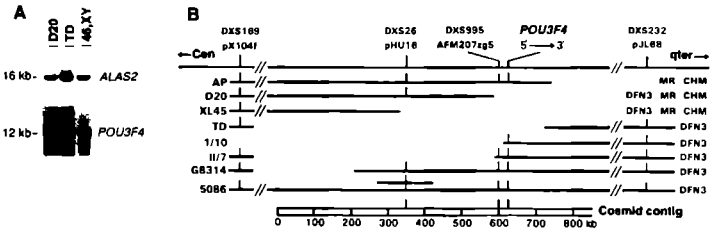


Fig. 1. Localization of the human *POU3F4* gene. (**A**) Southern blot analysis with the use of a mouse *Pou3f4* probe and a control probe. δ-aminolevulinate synthetase (*ALAS2*) with genomic DNAs from a control male, D20, and TD (*16*). Patient D20 has choroideremia (CHM), mental retardation (MR), and X-linked mixed deafness (DFN3); patient TD shows evidence of DFN3 (*12*). (**B**) Physical map of the DFN3 critical region. All patients with deletions have been described elsewhere (*12*). Patient 5086 carries a 150 kb duplication spanning DXS26. The extent of the cosmid contig is given at the bottom. *POU3F4* is located on cosmids IC2 (ICRFc104L0131) and IC3 (ICRFc104B1939), approximately 20 kb distal to DXS995 (*12*). Restriction mapping of the cosmid containing the *POU3F4* gene indicated that the *POU3F4* gene is oriented with its 5′ end toward the centromere.

Y. J. M. de Kok, S. M. van der Maarel, I. Huber, H. H. Ropers, F. P. M. Cremers, Department of Human Genetics, University Hospital Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, Netherlands.
M. Bitner-Glindzicz, S. Malcolm, M. E. Pembrey, Institute of Child Health, University of London, London WC1N 1EH, UK.
A. P. Monaco, University of Oxford, Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK.

*The first two authors contributed equally to this work.
†To whom correspondence should be addressed.

interval The rat homolog of *POU3F4*, *RHS2* is expressed during embryonic development in the brain, the neural tube, and the otic vesicle at 15 5 and 17 5 days after conception (*15*)

Thus, both its map position and its temporal and spatial expression pattern in early embryogenesis rendered *POU3F4* an attractive candidate gene for DFN3 To confirm and refine the localization of the human *POU3F4* gene, we amplified a murine genomic *Pou3f4* gene fragment by polymerase chain reaction (PCR) and hybridized it to Southern (DNA) blots containing Eco RI–digested DNA from patients with Xq21 deletions (*16*) A 12 kb Eco RI fragment was seen in the male control but not in the DFN3 patients TD and D20 who carry variably sized deletions in Xq21 (Fig 1A) The deletions overlap in a small chromosomal segment encompassing DXS995 (Fig 1B), which positions the *POU3F4* gene in a 120-kb region of the previously constructed 850 kb cosmid contig that spans the DFN3 locus By hybridizing the *Pou3f4* probe to cosmids from this contig, we could localize the *POU3F4* gene 20 kb distal to DXS995 (Fig 1B)

We used PCR primers complementary to the murine *Pou3f4* gene sequences to amplify a human *POU3F4* fragment from cosmid DNA, which was then used as a probe to screen a human fetal brain complementary DNA (cDNA) library (*17*) Six overlapping cDNAs were identified and partially characterized In total, we isolated 1 4 kb of the human *POU3F4* cDNA sequence, which contained the complete protein coding region of 1083 base pairs as inferred from the rat *RHS2* and mouse *Pou3f4* sequences (*15, 18, 19*) (Fig 2) The rat and mouse proteins are completely identical, and the human protein contains only four conservative amino acid substitutions (Fig 2)

To search for mutations in the *POU3F4* gene, we examined DNA from 14 unrelated patients with X-linked deafness and 50 unrelated control females from different ethnic origins for single-strand conformation (SSC) variants (*20*) Because the *POU3F4* gene contains no introns, five different PCR primer sets were sufficient to span the entire coding region of *POU3F4* Six of the deafness patients (Table 1) had characteristic features of DFN3—that is, a bony defect observed during CT scanning or a perilymphatic gusher encountered upon stapedectomy (*4–7*) In one case (patient 5823), audiologic data were consistent with a progressive mixed type of deafness, but neither CT scan nor stapes surgery were performed SSC shifts indicative of sequence alterations were found in four DFN3 patients, two of which are shown in Fig 3A, but not in the 50 control females

Subsequently, we sequenced the protein coding region of the *POU3F4* gene in all patients with X-linked hearing impairment In all four patients with SSC shifts as well as in patient 5823, who was not studied by PCR-SSC analysis, mutations were observed in the *POU3F4* gene In three DFN3 patients, we found small deletions in the POU specific and POU homeodomains of *POU3F4* that would result in frameshifts and

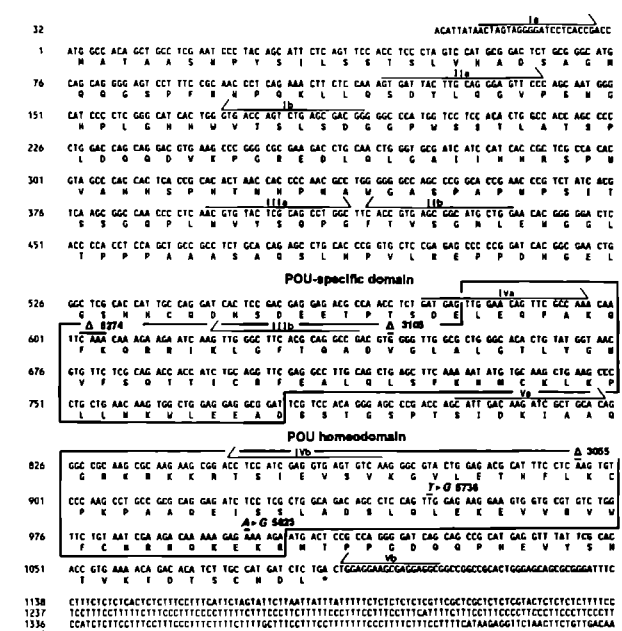


Fig. 2. Nucleotide sequence and the deduced amino acid sequence encoded by human *POU3F4* (*21*) The oligonucleotides used for PCR SSC analysis are indicated with arrows The POU specific and POU homeodomains are boxed The human POU3F4 protein is identical to the protein in mice and rats except for the following conservative amino acid changes from humans to rats and mice T15S P122S A159T and T356A The nucleotide changes and deletions (Δ) in the DFN3 patients are indicated, affected nucleotides are marked with a bar The asterisk indicates a stop codon

Table 1 Clinical features and *POU3F4* mutations of patients with X linked deafness (*21*) The deafness type here is based on audiologic examinat on The *POU3F4* mutations are named stop according to the last wild type amino acid that remains in the predicted protein Blank spaces indicate that no CT scan (second column) or stapedectomy (third column) was performed – designates that no mutations were found in *POU3F4*

Patient	Bony defect	Perilymph gusher	Deafness type	*POU3F4* mutation	Reference
2412		Yes	Mixed	–	(29)
2540	Yes	Yes	Mixed	–	(5)
3055		Yes	Mixed	L298 stop	(30)
3105	Yes	Yes	Mixed	D215 stop	(30)
5274	Yes		Sensorineural	K202 stop	(9)
5736	Yes		Mixed	L317W	(9)
5823*			Mixed	K334E	(30)
2418			Sensorineural	–	(30)
2964			Sensorineural	–	(30)
3036			Sensorineural	–	(30)
3056		No	Sensorineural	–	(30)
5106	No		Sensorineural	–	(9)
5347	No		Sensorineural	–	(9)
5943	No		Sensorineural	–	(9)
2266			Conductive	–	(30)

Some DFN3 affected maternal relatives of patient 5823 also have cataracts at a young age

premature stops of translation (Table 1 and Fig. 2). Patient 3055 carries a deletion of an A nucleotide at position 895 (Figs. 2 and 3C), patient 3105 has a deletion of one G that is part of a GGGG tetranucleotide stretch at positions 648 to 651, and in patient 5274 a CAAA tetranucleotide is deleted that is present in tandem at positions 603 to 610 of the wild-type *POU3F4* sequence. As shown in Fig. 3B by SSC analysis, the mutation found in patient 5274 cosegregates with the DFN3 phenotype in the whole family. Two patients had missense mutations in the *POU3F4* gene. In patient 5736, we found a T to G transversion at position 950, whereas patient 5823 showed an A to G transition at nucleotide 1000. These mutations result in the nonconservative L317W and K334E substitutions in the POU homeodomain (*21*).

The leucine residue at position 317 that is mutated in patient 5736 is located between helices 2 and 3 of the POU homeodomain, as deduced from nuclear magnetic resonance and crystallographic studies (*22*). It is conserved in six of seven members of the non-POU domain proteins that belong to the homeodomain superfamily (*23*) and in all POU domain proteins, except Oct-2 and emb. Here, the leucine residue is substituted for a methionine and a tyrosine residue, respectively (*24*). The wild-type lysine residue at position 334 that is mutated in patient 5823 resides in helix 3 of the POU homeodomain. This residue is conserved among all POU domain proteins and members of the homeodomain superfamily (*23, 24*) and contacts the DNA backbone in the major groove in the proposed crystal structure of the engrailed homeodomain-DNA complex, but not in the Oct-1 POU domain–octamer complex (*22, 25*). Thus, these data strongly suggest that all mutations we observed in the *POU3F4* gene are clinically relevant and give rise to DFN3. Because DFN3 is a rare disorder, the total number of DFN3 patients that can be investigated will remain limited. Nevertheless, the identification of five mutations in seven DFN3 patients as compared to no single mutation in 100 control X chromosomes is statistically highly significant (*26*).

Unexpectedly, three Xq21 microdeletions (G8314, II/7, and I/10) and one duplication (in patient 5086) that had been identified previously in patients with DFN3 do not encompass the *POU3F4* gene. In all four cases, the rearrangement is located proximal and 5′ to *POU3F4*, with physical distances varying between 15 and 400 kb (Fig. 1B). In none of these patients, nor in two others with either a perilymphatic gusher during stapes surgery or a temporal bone defect (patients 2412 and 2540), were point mutations detected in the *POU3F4* gene. Thus, in these cases DFN3 may be caused by mutations that affect 5′ noncoding or regulatory sequences mapping farther upstream. Alternatively, these aberrations may affect the gross chromosomal structure and thus may affect expression of *POU3F4*. Another, less likely explanation might be the presence of other genes in Xq21.1 that can cause DFN3.

In seven patients with X-linked sensorineural deafness and in one with X-linked conductive deafness, mutations were not found in the *POU3F4* gene. This is not surprising because previous linkage studies had indicated that X-linked deafness is heterogeneous. In at least one family with sensorineural deafness, the defect has been excluded from Xq21 (*9*). In two other families, linkage to the Xq21 and Xp11.3-p21.1 regions has been found (*27*). Here, we demonstrate that DFN3 is correlated with mutations that affect the POU3F4 protein. At least five POU domain genes are expressed in different parts of the developing inner ear (*28*), and defects of POU domain genes may play a major role in the etiology of nonsyndromic hearing impairment.

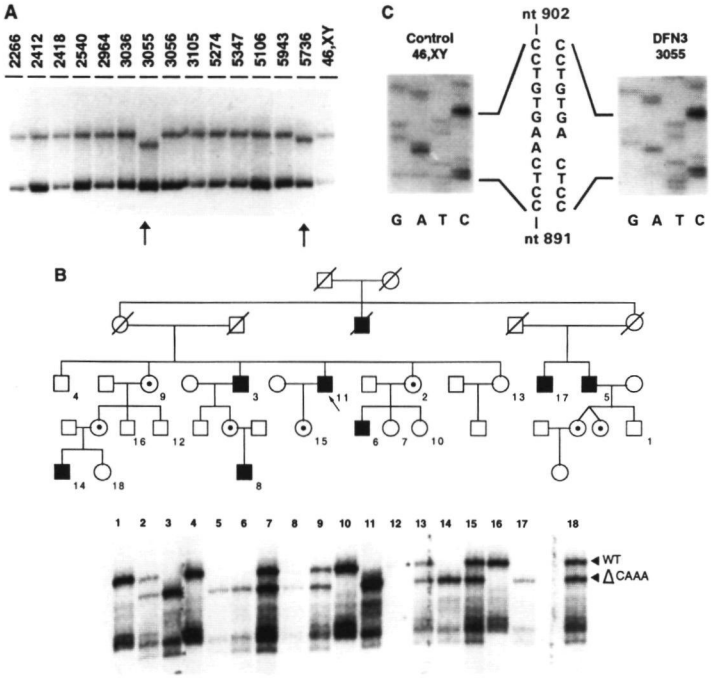


Fig. 3. Mutation analysis of *POU3F4*. (**A**) PCR-SSC analysis of the homeodomain of *POU3F4* in 14 patients with X-linked deafness and a male control (46, XY) with the use of the primer set V (*20*). The observed SSC shifts are indicated by arrows. (**B**) PCR-SSC analysis of the DFN3 family of patient 5274 (indicated by an arrow) with the use of primer set IV. Obligate carrier females are marked as such in the pedigree (circles with dots). DFN3 patients are indicated with solid squares; symbols with lines designate deceased individuals. The asterisk indicates the presence of a weak wild-type (WT) SSC band in lane 12. Of the four females at risk of carrying the CAAA deletion, individual 10 carries the wild-type *POU3F4* sequence, whereas individuals 7, 13, and 18 carry the mutation. (**C**) Nucleotide (nt) sequence of part of the POU homeodomain of patient 3055 and a control male.

REFERENCES AND NOTES

1. N. E. Morton, *Ann. N.Y. Acad. Sci.* **630**, 16 (1991).
2. L. Bergstrom, W. G. Hemenway, M. P. Downs, *Otolaryngol. Clin. N. Am.* **4**, 369 (1971).
3. P. E. Leon, H. Raventos, E. Lynch, J. Morrow, M.-C. King, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5181 (1992); P. Coucke *et al.*, *N. Engl. J. Med.* **331**, 425 (1994); P. Guilford *et al.*, *Nature Genet.* **6**, 24 (1994); P. Guilford *et al.*, *Hum. Mol. Genet.* **3**, 989 (1994); T. B. Friedman *et al.*, *Am. J. Hum. Genet.* **55**, abstr. 68 (1994).
4. W. E. Nance *et al.*, *Birth Defects* **7**, 64 (1971).
5. C. W. R. J. Cremers *et al.*, *Arch. Otolaryngol.* **111**, 249 (1985).
6. M. E. Glasscock, *ibid.* **98**, 82 (1973).
7. P. D. Phelps, W. Reardon, M. E. Pembrey, S. Bellman, L. Luxon, *Neuroradiology* **33**, 326 (1991).
8. H. G. Brunner *et al.*, *Hum. Genet.* **80**, 337 (1988); C. Wallis *et al.*, *Genomics* **3**, 299 (1988).
9. W. Reardon *et al.*, *Genomics* **11**, 885 (1991).
10. I. Bach *et al.*, *Hum. Genet.* **89**, 620 (1992); F. P. M. Cremers *et al.*, *Genomics* **4**, 41 (1989).
11. I. Bach *et al.*, *Am. J. Hum. Genet.* **50**, 38 (1992); C. Piussan *et al.*, *ibid.*, in press.
12. I. Huber *et al.*, *Hum. Mol. Genet.* **3**, 1151 (1994).
13. Y. J. M. de Kok *et al.*, unpublished results.
14. P. J. Douville, S. Atanasoski, A. Tobler, A. Fontana,

M E Schwab *Mamm Genome* **5** 180 (1994)
15 C Le Moine and W S Young III, *Proc Natl Acad Sci U S A* **89** 3285 (1992)
16 Genomic DNAs (10 μg of each) were digested with Eco RI The fragments were separated by gel electrophoresis and blotted on GeneScreenPlus membranes (NEN) as recommended by the manufacturer The mouse *Pou3f4* genomic segment was amplified from 500 ng of mouse genomic DNA, 250 ng of the primers 977 (nucleotides 119 to 140 of the *POU3F4* open reading frame (ORF) (*19*) GTGAC TACTTGCAGGGAGTTCC] and 978 (nucleotides 521 to 540 of the ORF, GCAGTGGTGCGAGC CCAGCT Isogen Biosciences), 10 mM tris HCl (pH 8 0), 50 mM KCl, 3 mM $MgCl_2$ and 0 01% gelatin After initial denaturation for 5 min at 94°C, amplification was done in 30 cycles of 1 min at 94°C, 1 min at 64 C and 1 min at 72°C with a final elongation step for 6 min at 72°C The PCR product was purified from low melting temperature (LMT) agarose and radiolabeled with the use of random hexamer priming The blot in Fig 1A was hybridized in 0 5 M NaH_2PO_4 (pH 6 8), 7% (w/v) SDS 1 mM EDTA and 50 μg/ml of sonicated, denatured herring sperm DNA at 55°C for 18 hours Washing was done in 40 mM NaH_2PO_4 and 1% (w/v) SDS at 60°C for 1 hour The mouse *Alas2* cDNA probe pMS20 [D S Schoenhaut and P J Curtis, *Gene* **48**, 55 (1986)] was hybridized to the blot shown in Fig 1A, as described for the mouse *Pou3f4* probe The gene encoding human ALAS2 is located in Xp11 2 [P D Cotter H F Willard, J L Gorski, D F Bishop *Genomics* **13**, 211 (1992)]
17 We used the mouse *Pou3f4* primers 977 and 978 (*16*) to amplify a 420-bp segment of the human *POU3F4* gene using 10 ng of cosmid IC3 The PCR product was purified in an LMT agarose gel and labeled as described (*16*) Phage recombinants (10^6) of a fetal brain cDNA library (Stratagene) were screened with this DNA fragment as described [F P M Cremers, D J R van de Pol L P M van Kerkhoff B Wieringa, H H Ropers *Nature* **347** 674 (1990)] Six cDNA clones were isolated, four of which were analyzed further The ORF of the human *POU3F4* gene was sequenced on both strands with the use of mouse and human *POU3F4* oligonucleotides that amplified DNA from cosmid IC3 and DNA from the four cDNA clones Sequences from the 5′ and 3′ untranslated regions were obtained from the cDNA clones Sequence analysis was performed with the dye terminator sequencing kit on an Applied Biosystems automated sequencer The human *POU3F4* cDNA sequence has been deposited in the European Molecular Biology Laboratory database with accession number X82324
18 Y Hara, A C Rovescalli, Y Kim, M Nirenberg, *Proc Natl Acad Sci U S A* **89** 3280 (1992)
19 J M Mathis *et al EMBO J* **11** 2551 (1992)
20 PCR SSC analysis [M Orita Y Suzuki T Sekiya K Hayashi *Genomics* **5** 874 (1989)] was performed with five partially overlapping PCR products that span the ORF of *POU3F4* We added 250 ng of each primer from the five sets of primers [Ia (ACTAGTAGGGGATCCTCACCG) and Ib (CCGTCGCTCAGACTGGTCAC) IIa (GTGACTACTTGCAGGGAGTTCC) and IIb (CCAGCATACCGCTCACCGTG) IIIa (ACGTGTACTCGCAGCCTGGC) and IIIb (CGGTCTGCGTGAAGCCCAAC) IVa (GATGAGTTGGAACAGTTCGCCAA) and IVb (TGACACTCACCTCGATGGAGG) and Va (CATTGACAAGATCGCTGCAC) and Vb (GCCTCCTCGCTTCCTCCA)] to buffers containing either 10 mM tris-HCl (pH 8 0), 50 mM KCl, 3 mM $MgCl_2$ and 0 01% gelatin (primer set I), 10 mM tris-HCl (pH 8 0) 50 mM KCl 2 mM $MgCl_2$ and 1 mM dithiothreitol (primer sets II IV and V) or 30 mM tricine HCl (pH 8 4), 2 mM $MgCl_2$, 0 01% gelatin, and 5 mM β mercaptoethanol (primer set III), supplemented with 500 ng of genomic DNA 4 mM deoxyadenosine triphosphate deoxyguanosine triphosphate and deoxythymidine triphosphate (dTTP) 1 mM deoxycytidine triphosphate (dCTP), 1 μCi of [α-^{32}P]dCTP and 1 unit of Taq DNA polymerase (Boehringer) After initial denaturation for 5 min at 94°C amplification was done in 30 cycles of 1 min at 94°C 1 min at 64°C (primer sets I through IV), or 1 min at 58°C (primer set V) and 1 min at 72°C with a final elongation step for 6 min at 72°C The PCR products were separated on a 6% polyacrylamide gel, with or without 10% (w/v) glycerol, for 4 hours at 35 W at 4°C After vacuum drying the gels were exposed to Xomat films (Kodak) for 16 hours at room temperature The sequence of PCR products was established for both strands as described (*17*) Primers IIa IIb, and IIIb were deduced from mouse *Pou3f4* sequences The nucleotides that do not match the human *POU3F4* sequence are underlined above The sequence shown in Fig 3B was established with the use of ^{32}P end-labeled primer Va with the cycle sequencing kit (Boehringer)
21 Mutations are indicated with the single-letter amino acid abbreviations, thus, $Leu^{317} \rightarrow Trp$ is indicated by L317W Abbreviations for the amino acid residues are A, Ala C, Cys, D Asp E, Glu, F, Phe, G, Gly, H, His, I, Ile, K Lys L Leu M Met, N, Asn P, Pro, Q, Gln, R, Arg, S Ser, T, Thr, V, Val, W, Trp, and Y, Tyr
22 J D Klemm *et al*, *Cell* **77**, 21 (1994)
23 C A Pabo and R T Sauer, *Annu Rev Biochem* **61**, 1053 (1992)
24 K Okamoto *et al*, *J Biol Chem* **268** 7449 (1993)
25 C R Kissinger *et al*, *Cell* **63**, 579 (1990)
26 This was determined by the two-sided Fisher's exact test, $P < 0.001$
27 A Lalwani *et al*, *Am J Hum Genet* **53**, 1027 (1993), M Bitner-Glindzicz *et al*, *J Med Genet* **31**, 916 (1994)
28 A F Ryan E B Crenshaw III D M Simmons *Ann N Y Acad Sci* **630**, 129 (1991)
29 P Thorpe *et al S A Med J* **48** 587 (1974)
30 Personal communications of K Shaver-Arnos (patients 3055 and 3056) O Michel (patient 3105) I van Langen and R Hennekam (patient 5823), H G Brunner (patients 2266 and 2418), M Stul (patient 2964), and J Gouldsmith (patient 3036)
31 The authors are indebted to P Beighton, J Gouldsmith R Hennekam, I van Langen, O Michel, W E Nance, W P Reardon, N Stul, K Shaver Arnos and P Thorpe for providing blood and information of patients with X-linked deafness We are especially grateful to H G Brunner for his support of this project We thank I H J M Scholten, S D van der Velde-Visser, and E Boender-van Rossum for excellent technical assistance and J Hendriks for statistical analysis We thank Y Ishikawa-Brush for YAC screening and M Ross, G Zehetner, and H Lehrach for access to the Imperial Cancer Research Fund cosmid library filters and the Reference Library Database London The research of F P M C has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences M B G is a Medical Research Council Clinical Training Fellow M E P is funded by Mothercare This work was supported by the Netherlands Organization for Scientific Research (NWO) by the European Community and by the Imperial Cancer Research Fund

22 September 1994 accepted 28 December 1994

CHAPTER 5

A DUPLICATION/PARACENTRIC INVERSION ASSOCIATED WITH FAMILIAL X-LINKED DEAFNESS (DFN3) SUGGESTS THE PRESENCE OF A REGULATORY ELEMENT MORE THAN 400 KB UPSTREAM OF THE POU3F4 GENE

de Kok, Y J, Merkx, G F, van der Maarel, S M, Huber, I, Malcolm, S, Ropers, H -H, and Cremers, F P M

Hum Mol Genet., **4**, 2145-2150 (1995).

A duplication/paracentric inversion associated with familial X-linked deafness (DFN3) suggests the presence of a regulatory element more than 400 kb upstream of the *POU3F4* gene

Yvette J M.de Kok*, Gerard F.M Merkx, Silvère M van der Maarel, Irene Huber, Susan Malcolm[1], Hans-Hilger Ropers and Frans P M.Cremers

Department of Human Genetics University Hospital Nijmegen Geert Grooteplein Zuid 20 6525 GA Nijmegen The Netherlands and [1]Institute of Child Health University of London 30 Guilford Street London WC1N 1EH UK

Received July 10 1995 Revised and Accepted August 24 1995

X-linked deafness with stapes fixation (DFN3) is caused by mutations in the *POU3F4* gene at Xq21.1. By employing pulsed field gel electrophoresis (PFGE) we identified a chromosomal aberration in the DNA of a DFN3 patient who did not show alterations in the open reading frame (ORF) of *POU3F4*. Southern blot analysis indicated that a DNA segment of 150 kb, located 170 kb proximal to the *POU3F4* gene, was duplicated. Fluorescence *in situ* hybridization (FISH) analysis, PFGE, and detailed Southern analysis revealed that this duplication is part of a more complex rearrangement including a paracentric inversion involving the Xq21.1 region, and presumably the Xq21.3 region. Since at least two DFN3-associated minideletions are situated proximal to the duplicated segment, the inversion most likely disconnects the *POU3F4* gene from a regulatory element which is located at a distance of at least 400 kb upstream of the *POU3F4* gene.

INTRODUCTION

The most frequent form of X-linked deafness, DFN3, is characterized by fixation of the stapes and a perilymphatic gusher upon stapedectomy By employing computerized tomography, Phelps *et al* (1) identified a unique abnormality of the petrous temporal bone consisting of a dilatation of the internal auditory meatus (IAM) and an abnormal wide communication between the basal turn of the cochlea and the IAM The gene underlying DFN3 was mapped to Xq21 by linkage analysis and through molecular characterization of large deletions associated with choroideremia, mental retardation, and DFN3 (2–7) Smaller deletions were found in five patients with typical DFN3 (8–11) Yeast artificial chromosome (YAC) clones that span the critical region were isolated, and an 850 kb cosmid contig was constructed (9)

Recently, Douville *et al* (12) assigned a mouse POU domain gene, *Brain 4*, to a region of the murine X chromosome that is homologous to human Xq13–q22 The rat homolog of *Brain 4*, *RHS2*, is expressed during embryonic development in the brain, the neural tube, the whisker roots, and the otic vesicle (13) We cloned and characterized the human homolog of *Brain 4*, *POU3F4*, and were able to position the gene in the critical region for DFN3 In seven unrelated patients with DFN3 but not in 100 control X chromosomes, small mutations were found in the *POU3F4* gene that result in truncation of the predicted protein or in non-conservative amino acid substitutions (14,15) Surprisingly, the intronless *POU3F4* gene was found to be located up to 400 kb distal to four minideletions associated with typical DFN3 (11,14)

Here, we describe the identification and detailed characterization of another, more complex chromosomal rearrangement, a duplication/paracentric inversion associated with familial DFN3 We show that one of the inversion breakpoints is situated 320 kb proximal to *POU3F4* Since at least two DFN3 associated minideletions reside proximal to the duplication, this finding suggests that the expression of *POU3F4* is regulated by a sequence element located several hundred kilobases farther proximal

RESULTS

Identification of a duplication associated with DFN3 by PFGE and Southern blot analysis

We performed pulsed field gel electrophoresis analysis of genomic DNA of several DFN3 patients using a cosmid (4893F6, Fig 5) located in the Xq21 1 region proximal to the *POU3F4* gene In the DNA of DFN3 patient 5086, we observed a *Sfi*I fragment of 575 kb instead of the normal 675 kb, suggestive of a microdeletion or a *Sfi*I restriction fragment length polymorphism (Fig 1a) Southern blot analysis of *Eco*RI digested DNA of patient 5086 employing all cosmids from a previously established 850 kb contig did not yield a deletion In contrast, the hybridization signals of several cosmids near the DXS26 locus suggested the presence of a duplicated DNA fragment (data not shown) To investigate this possibility in more detail, we constructed *Eco*RI and *Taq*I blots containing equal amounts of DNA from a control female, a control male, DFN3 patient 5086, and patient XL45 The latter patient carries a microscopically visible deletion com-

*To whom correspondence should be addressed

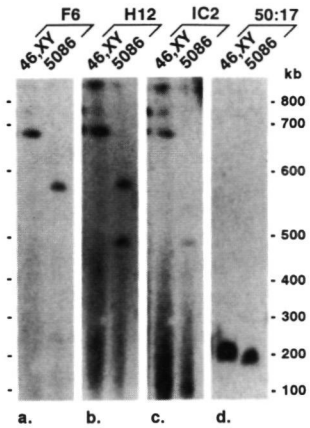


Figure 1. PFGE analysis of *Sfi*I digested high-molecular-weight DNA from a male control and DFN3 patient 5086. Hybridization results are shown for cosmid 4893F6 **(a)**, cosmid 4893H12 **(b)**, cosmid IC2 **(c)** and for a control probe 50:17 **(d)**, located in Xq13.1 (van der Maarel *et al.* manuscript in preparation). The scale shown on the right is based on the migration of the *Saccharomyces cerevisiae* chromosomes which were employed as size markers.

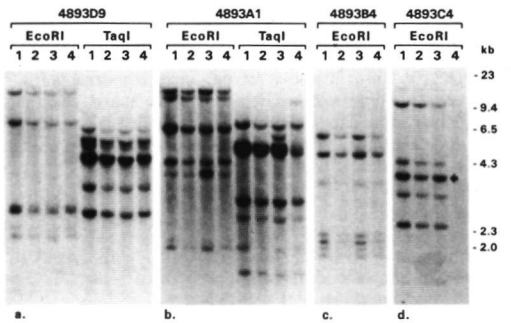


Figure 2. Southern blot analysis of genomic DNAs from a control female (lane 1), control male (lane 2), DFN3 patient 5086 (lane 3), and the syndromic DFN3 patient XL45 (lane 4). The restriction enzymes and hybridization probes used are indicated on top of each panel. **(a)** Cosmid 4893D9 detects restriction fragments of equal intensity in patient 5086 and the control male. **(b)** Cosmid 4893A1 detects signals of normal and double intensity as well as aberrantly sized *Eco*RI (4.0 kb) and *Taq*I (6.0 kb) fragments in patient 5086. **(c)** Cosmid 4893B4 detects signals with a double intensity in patient 5086 compared to a male control. **(d)** Cosmid 4893C4 detects one fragment (indicated with an arrow) which is either of double intensity compared to the male control lane, or represents a novel aberrantly sized fragment. Size markers are given on the right.

mencing in the region investigated here. In patient 5086, a double intensity of *Eco*RI and *Taq*I bands compared to control male DNA was observed for cosmids 4893B4 (Fig. 2c), 4893K6, 4893B5, 4893K2, and 4893H12 (Fig. 5; Southern

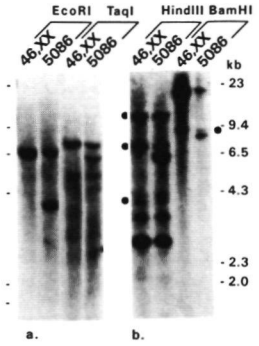


Figure 3. Molecular characterization of the duplication/inversion endpoints in patient 5086. Southern blot analysis of genomic DNAs digested with the enzymes given above the lanes using a 6.5 kb *Eco*RI fragment of cosmid 4893A1 **(a)** and a 1.7 kb *Eco*RI fragment of cosmid 4893C4 **(b)**. Black dots mark the signals from a previous hybridization. Size markers are given on the right.

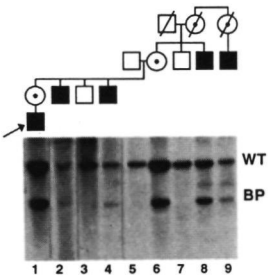


Figure 4. Southern blot analysis of *Eco*RI digested DNA from patient 5086 (arrow) and several of his relatives. The 6.5 kb *Eco*RI fragment of cosmid 4893A1 was employed as a probe. WT = wildtype; BP = breakpoint.

blot data not shown). Cosmid 4893A1 detects restriction fragments with a normal intensity, a double intensity, as well as an aberrantly sized *Eco*RI (4.0 kb) and *Taq*I (6.0 kb) fragment (Fig. 2b). All cosmids proximal to 4893A1 showed normal hybridization results, as illustrated by cosmid 4893D9 (Fig. 2a). For cosmid 4893C4, only a 4.0 kb *Eco*RI band appeared to be of double intensity compared to control male DNA (Fig. 2d); no aberrantly sized fragments were detected. All cosmids distal to 4893C4 showed normal hybridization results (data not shown).

Detailed mapping of the duplication endfragments and family analysis

The endfragments of the duplicated segment in patient 5086 were mapped in detail by analysing genomic DNA from patient 5086 and an unaffected control individual with *Eco*RI fragments from cosmids 4893A1 and 4893C4. At the proximal end, a 6.5 kb *Eco*RI fragment of cosmid 4893A1 detected 4.0

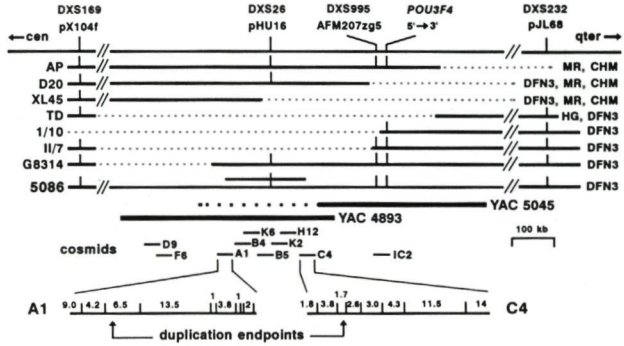


Figure 5. Physical map of the DFN3 critical region. All patients with deletions except AP have been described elsewhere (9,20). Patient AP shows choroideremia (CHM) and mental retardation (MR) (20). Deletions are indicated by stippled lines. The duplicated segment in patient 5086 is depicted by a double bar. *Eco*RI maps of the cosmids spanning the duplication breakpoints are given at the bottom. *Eco*RI fragment sizes are given in kilobases. Only a subset of the cosmids from the contig is depicted. DFN3 = X-linked deafness with stapes fixation; HG = hypogonadism.

kb *Eco*RI and 6.0 kb *Taq*I breakpoint fragments (Fig. 3a). Since we were unable to identify aberrantly sized fragments with cosmid 4893C4 in *Eco*RI and *Taq*I digested DNA of patient 5086, blots were constructed containing *Hin*dIII and *Bam*HI digested DNAs. The distal endpoint of the duplication could be detected with a 1.7 kb *Eco*RI fragment from cosmid 4893C4 which, in addition to normally sized restriction fragments, clearly hybridizes to novel *Hin*dIII and *Bam*HI fragments (Fig. 3b). Based on the signal intensities observed, the distal duplication breakpoint is located in a 2.8 kb *Hin*dIII and a 18 kb *Bam*HI fragment. From the previously established *Eco*RI restriction map of the cosmid contig, we estimate that the duplicated DNA segment measures 150 kb (Fig. 5).

To investigate whether this rearrangement segregates with the DFN3 phenotype in the family of patient 5086, we hybridized the 6.5 kb *Eco*RI fragment of cosmid 4893A1 to a Southern blot containing *Eco*RI digested DNAs from several family members. Clearly, the breakpoint fragment indicative of the chromosomal rearrangement can be seen in all DFN3 patients and female carriers (Fig. 4).

FISH and PFGE analysis

To investigate whether the two copies of the duplicated segment are located next to each other near the *POU3F4* gene, a cosmid located on the duplicated segment, 4893H12, was hybridized to metaphase chromosomes prepared from an EBV-immortalized lymphoblastoid cell line of patient 5086. In most chromosome spreads, the X-chromosome showed two specific, but rather diffuse signals (Fig. 6a). To our surprise, we observed four discrete signals in approximately one-third of the metaphases investigated, indicating that cosmid 4893H12 hybridizes to two different regions of the X-chromosome of patient 5086 (Fig. 6b,c). In a control metaphase X-chromosome, 4893H12 identified one distinct locus in the Xq21 band (data not shown). The most straightforward explanation for the FISH and Southern blotting results is a duplication-paracentric inversion event which moved one of the copies of the duplicated segment away from the endogenous copy of cosmid 4893H12 (Fig. 7).

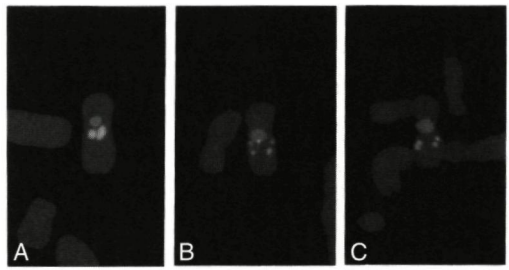


Figure 6. FISH analysis of three X-chromosomes in a metaphase chromosome spread of EBV-immortalized peripheral blood cells of patient 5086 using the centromere X probe (pBAMX5), detected in red, in conjunction with probe 4893H12, detected in yellow/green. Chromosomes are counterstained using the blue dye DAPI.

To test this hypothesis, cosmid 4893H12 and cosmid IC2, the latter of which contains the *POU3F4* gene, were successively employed as probes on the PFGE blot described above. As expected, cosmid 4893H12 detected the 575 kb *Sfi*I fragment identified by cosmid 4893F6, corresponding to one of the inversion breakpoints, and an additional 475 kb *Sfi*I fragment (Fig. 1b). Since the latter fragment is also identified by cosmid IC2 (Fig. 1c), it most likely spans the other inversion-breakpoint as indicated in Figure 7. To investigate whether the inversion involves chromosomal sequences proximal or distal to the *POU3F4* gene, FISH analysis was performed with differently labeled cosmids from the duplication (4893H12) and the choroideremia (*CHM*) gene (cosmid U98B5) located at Xq21.2. In only one X-chromosome, the *CHM* cosmid could be localized between the duplicated sequences; in all other metaphase chromosome spreads analysed, the 4893H12 and U98B5 signals were not resolved (data not shown).

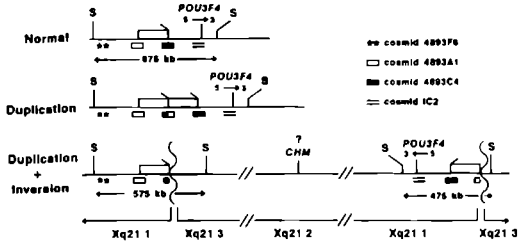


Figure 7 Model for the duplication/paracentric inversion event that gave rise to the observed rearrangement in DFN3 patient 5086 The upper drawing reflects the normal situation the middle shows an intermediate structure resulting from a DNA duplication event the lower schematic illustrates the proposed final rearrangement observed in patient 5086 The locations of the *Sfi*I (S) sites flanking the duplicated segment were derived from the cosmid contig (unpublished data) The locations of the *Sfi*I sites in the Xq21 3 region were deduced from the observed *Sfi*I fragment sizes (see Fig 1)

POU3F4 analysis in patient 5086

DNA of patient 5086 was examined for single strand conformation (SSC) variants by employing PCR primer sets defining five overlapping DNA segments that span the entire coding sequence of the *POU3F4* gene (14) All five DNA segments could be readily amplified indicating no apparent structural abnormality in the protein coding region of *POU3F4* No SSC shifts indicative of sequence alterations were found The entire ORF of *POU3F4* was analysed by DNA sequencing but no abnormalities were found

DISCUSSION

We have identified and characterized a complex rearrangement in a patient with DFN3 The results of PFGE and Southern blot analysis are not consistent with a simple tandem duplication event, i e insertion of the new copy adjacent to the endogenous sequence proximal to the *POU3F4* gene Since *Sfi*I restriction sites are known to flank the duplicated segment (Fig 7) this event would generate a 825 kb *Sfi*I band and not the observed 575 kb *Sfi*I band FISH analysis using a cosmid from the duplicated segment showed that the duplicated segments are separated by several megabases of DNA Results of PFGE and Southern blotting are compatible with a duplication/paracentric inversion involving sequences at Xq21 1 near *POU3F4* and Xq13 3 or Xq21 3 In agreement with this, cosmid 4893H12, located on the duplicated segment, and cosmid IC2 spanning the *POU3F4* gene detected an additional *Sfi*I fragment corresponding to the other breakpoint of the inversion (Fig 1b c) FISH analysis suggests that the *CHM* gene at Xq21 2 is located between the duplicated segments Also, positioning of the duplicated segments along the X-chromosome suggests locations at Xq21 1 and Xq21 3, although the involvement of the Xq13 band cannot be ruled out completely Both high resolution metaphase FISH analysis as well as detailed PFGE analysis using cosmids distal to the *CHM* gene will enable the precise positioning of this breakpoint

The duplication/inversion event proposed here does not affect the *POU3F4* gene proper which, in the rearranged situation (Fig 7), is situated 170 kb proximal to one copy of the duplicated segment and 320 kb proximal to the distal inversion breakpoint To explain the DFN3 phenotype in patient 5086, as well as the aforementioned minideletions situated proximal to the *POU3F4* gene, a few possibilities are considered First, in the normal situation, the 5′ part of the *POU3F4* gene, including its promoter, might be situated farther centromeric In this situation, the *POU3F4* gene would contain a single unusually large intron (>400 kb) in its 5′ untranslated region (Fig 5) We have located the 3′ end of the *POU3F4* mRNA 2 4 kb downstream of the ORF Since the *POU3F4* mRNA was estimated to be 3 5 kb in size we can deduce that the 5 untranslated region measures less than 200 bp (unpublished data) From these findings we deduce that most probably there is no large 5′ intron in the *POU3F4* gene Second, another gene involved in DFN3 might be situated in the chromosomal segment proximal to *POU3F4* If so, this gene would be predicted to span a region of more than 200 kb the distance between the proximal inversion-breakpoint observed in patient 5086 and the DFN3 associated deletion mapping farthest centromeric (G8314 Fig 5) We believe that this is an unlikely possibility, too since this chromosomal segment was found to be devoid of sequences transcribed in fetal brain tissue In a third model the three minideletions and the duplication/inversion would juxtapose heterochromatic sequences from Xq21 1 and Xq21 3 respectively, near the *POU3F4* gene, which then might down regulate transcription of the *POU3F4* gene In *D melanogaster* translocation of genes into heterochromatin is known to give rise to cell autonomous gene silencing a phenomenon called position effect variegation (16) In humans examples of position effects are rare Campomelic dysplasia a skeletal malformation syndrome and autosomal sex reversal is not only caused by mutations in the *SOX9* gene, but also by translocations involving sequences located more than 50 kb away from the *SOX9* gene (17 18) Similarly translocations up to 85 kb from the *PAX6* gene are causally related to the aniridia phenotype (19) If this mechanism plays a role in the Xq21 region, it is directional in nature, since a large deletion 120 kb distal to the *POU3F4* gene (patient AP, Fig 4), is associated with mental retardation and choroideremia but not with hearing impairment (20) It remains to be investigated whether transcriptional silencing of the respective genes is due to position effects

To explain the DFN3 phenotype in patient 5086, we favour a model in which the proposed inversion separates a control element, most likely an enhancer element, from the *POU3F4* transcription unit A similar situation was reported for the α and β globin gene clusters in which deletions remove important control regions (21,22) To account for the clinical findings in all patients with minideletions [patients I/10, II/7, G8314 (Fig 4) and patient ML (11)] that do not span the *POU3F4* gene, the putative enhancer sequence should be located more than 400 kb upstream of the gene Since in none of these patients mutations were found in the ORF of the *POU3F4* gene (14, Y J M de K and F P M C , unpublished data), the deletions must be causative for the observed phenotype It is noteworthy that the deletion in patient II/7 is accompanied by a paracentric inversion The breakpoints in

this familial case are in Xq13.1 and Xq21.2 (23). Thus far, we were unable to test our hypothesis directly since reverse transcription-PCR analysis of the *POU3F4* mRNA isolated from control lymphoblast failed, indicating that POU3F4 expression in lymphoblasts is very low.

In three patients with DFN3 we were unable to find causative mutations in or outside the DFN3 gene. If the expression of this gene depends on the presence of an enhancer situated proximal to the *POU3F4* gene, small mutations or chromosomal abnormalities might be found in the chromosomal region centromeric to the cosmid contig. To investigate this region in more detail, a YAC clone from this particular region was recently isolated (20) and the construction of a cosmid contig is underway. Elucidation of the molecular mechanism responsible for the DFN3 phenotype in patients with structural abnormalities at a large distance from the *POU3F4* gene will yield important new insights into the regulation of this gene.

MATERIALS AND METHODS

DFN3 patients

Patient 5086 is the youngest member of a multigeneration deafness family (24, Fig. 4). Audiologic examination showed a profound sensorineural hearing loss. Two maternal uncles of the proband showed a total hearing loss. Radiological examination using computerized tomography in patient 5086 and two maternal uncles revealed dilated internal auditory canals and structural lesions of the cochlea that cause an incomplete separation of perilymphatic and cerebrospinal fluids. The mother of the proband showed a moderate mixed hearing loss in a pure tone audiogram (24). Together these findings suggest that the deafness in this family can be classified as DFN3. In most patients with DFN3, both sensorineural and conductive hearing loss is found. In this case the conductive element is probably masked by the profound sensorineural component. Patient XL45 suffers from DFN3, mental retardation and choroideremia, and has been described in more detail elsewhere (7,25,26).

Pulsed field gel electrophoresis and Southern blotting

PFGE and Southern blot analysis were performed as described by Bach *et al.* (7) and Huber *et al.* (9), respectively. Cosmid IC2 corresponds to the ICRF clone c104L0131. Clone 50.17 (DXS6673E) is a cDNA constituting part of a gene spanning an Xq13.1 translocation breakpoint which is possibly involved in mental retardation (Van der Maarel *et al.*, in preparation).

Single strand conformation analysis and nucleotide sequencing

Polymerase chain reaction-single strand conformation (PCR-SSC) analysis (27) was performed employing five partially overlapping PCR segments spanning the ORF of *POU3F4* as described elsewhere (14).

Fluorescence *in situ* hybridization

All fluorescence *in situ* hybridization (FISH) procedures used were essentially as described previously (28–30). Briefly, cosmid 4893H12 was labeled with dig-11-dUTP (Boehringer) and the X centromere probe pBAMX5 with Fluorolink Cy3-dCTP (BDS inc., Pittsburgh) using a nick translation kit (Gibco, Life Technologies). 100 ng labeled 4893H12 probe DNA and 5 µg Cot-1 DNA (Gibco Life Technologies) was dissolved in 6 µl of a hybridization solution (50% v/v deionized formamide, 10% w/v dextrane sulphate, 2×SSC, 1% v/v Tween 20, pH 7.0). Prior to hybridization the probe was denatured at 80°C for 10 min, chilled on ice and incubated at 37°C for 30 min allowing preannealing. For pBAMX5, 20 ng DNA in 6 µl was used per reaction and no competitor DNA was added. Metaphase spreads were prepared using standard procedures. After denaturation of the slides, probe incubations were carried out under an 18×18 mm coverslip in a moist chamber for 45 h.

Immunocytochemical detection of the hybridizing probes was achieved using FITC conjugated sheep anti-digoxigenin (1:20, Boehringer Mannheim). For evaluation of the chromosomal slides a Zeiss epifluorescence microscope equipped with appropriate filters for visualization of Texas Red, DAPI and FITC fluorescence was used. Digital images were acquired using a high performance cooled CCD camera (Photometrics, Tucson, USA), interfaced to a Macintosh IIci computer. All digital image-acquiring, -processing and analysis functions were accomplished by means of the BDS-Image™ FISH software package (Biological Detection Systems Inc., Rockville, USA).

ACKNOWLEDGEMENTS

The authors thank S.D. van der Velde-Visser and E. Boender-van Rossum for technical assistance in cell culturing. We thank Drs. M. Bitner-Glindzicz and M.E. Pembrey for helpful suggestions. The research of F.P.M.C. has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences. Y.J.M. de K. and I.H. were supported by the Netherlands Organization for Scientific Research (NWO).

REFERENCES

1. Phelps, P.D., Reardon, W., Pembrey, M., Bellman, S. and Luxon, L. (1991) X-linked deafness, stapes gushers and a distinctive defect of the inner ear. *Neuroradiology*, **33**, 326–330.
2. Rosenberg, T., Schwartz, M., Niebuhr, E., Yang, H.-M., Sardemann, H., Andersen, O. and Lundsteen, C. (1986) Choroideremia in interstitial deletion of the X chromosome. *Ophthalmic Paediatr. Genet.*, **7**, 205–210.
3. Cremers, F.P.M., van de Pol, T.J.R., Wieringa, B., Hofker, M.H., Pearson, P.L., Pfeiffer, R.A., Mikkelsen, M., Tabor, A. and Ropers, H.-H. (1988) Molecular analysis of male-viable deletions and duplications allows ordering of 52 DNA probes on proximal Xq. *Am. J. Hum. Genet.*, **43**, 452–461.
4. Cremers, F.P.M., van de Pol, T.J.R., Diergaarde, P.J., Wieringa, B., Nussbaum, R.L., Schwartz, M. and Ropers, H.-H. (1989) Physical fine mapping of the choroideremia locus using Xq21 deletions associated with complex syndromes. *Genomics*, **4**, 41–46.
5. Cremers, F.P.M., Sankila, E.M., Brunsmann, F., Jay, M., Jay, B., Wright, A., Pinckers, A.J.L.G., Schwartz, M., van de Pol, T.J.R., Wieringa, B., de la Chapelle, A., Pawlowitzki, I.H. and Ropers, H.-H. (1990) Deletions in patients with classical choroideremia vary in size from 45 kb to several megabases. *Am. J. Hum. Genet.*, **47**, 622–628.
6. Merry, D.E., Lesko, J.G., Sosnoski, D.M., Lewis, R.A., Lubinsky, M., Trask, B., van den Engh, G., Collins, F.S. and Nussbaum, R.L. (1989) Choroideremia and deafness with stapes fixation: A contiguous gene deletion syndrome in Xq21. *Am. J. Hum. Genet.*, **45**, 530–540.
7. Bach, I., Robinson, D., Thomas, N., Ropers, H.-H. and Cremers, F.P.M. (1992) Physical fine mapping of genes underlying X linked deafness and non fra(X) X linked mental retardation at Xq21. *Hum. Genet.*, **89**, 620–624.
8. Bach, I., Brunner, H.G., Beighton, P., Ruvalcaba, R.H.A., Reardon, W., Pembrey, M.E., van der Velde-Visser, S.D., Bruns, G.A.P., Cremers, C.W.R.J., Cremers, F.P.M. and Ropers, H.-H. (1992) Microdeletions in patients with gusher-associated X linked mixed deafness (DFN3). *Am. J. Hum. Genet.*, **50**, 38–44.
9. Huber, I., Bitner-Glindzicz, M., de Kok, Y.J.M., van der Maarel, S.M., Ishikawa-Brush, Y., Monaco, A.P., Robinson, D., Malcolm, S., Pembrey, M.E., Brunner, H.G., Cremers, F.P.M. and Ropers, H.-H. (1994) X linked mixed deafness (DFN3): cloning and characterization of the critical region allows the identification of novel microdeletions. *Hum. Mol. Genet.*, **3**, 1151–1154.
10. Piussan, C., Hanauer, A., Dahl, N., Mathieu, M., Kolski, C., Biancalana, V., Heyberger, S. and Strunski, V. (1995) X-linked progressive mixed deafness: a new microdeletion that involves a more proximal region in Xq21. *Am. J. Hum. Genet.*, **56**, 224–230.
11. Dahl, N., Laporte, J., Hu, L., Biancalana, V., Le Paslier, D., Cohen, D., Piussan, C. and Mandel, J.L. (1995) Deletion mapping of X-linked mixed deafness (DFN3) identifies a 265–525-kb region centromeric of DXS26. *Am. J. Hum. Genet.*, **56**, 999–1002.
12. Douville, P.J., Atanasoski, S., Tobler, A., Fontana, A. and Schwab, M.E. (1994) The brain-specific POU box gene *Brn4* is a sex-linked transcription factor located on the human and mouse X chromosomes. *Mamm. Genome*, **5**, 180–182.
13. le Moine, C. and Young, W.S., III (1992) *RHS2*, a POU domain-containing gene, and its expression in developing and adult rat. *Proc. Natl. Acad. Sci. USA*, **89**, 3285–3289.
14. de Kok, Y.J.M., van der Maarel, S.M., Bitner-Glindzicz, M., Huber, I., Monaco, A.P., Malcolm, S., Pembrey, M.E., Ropers, H.-H. and Cremers, F.P.M. (1995) Association between X-linked mixed deafness and mutations in the POU domain gene *POU3F4*. *Science*, **267**, 685–688.
15. Bitner-Glindzicz, M., Turnpenny, P., Hoglund, P., Kaariainen, H., Sankila, E.-M., van der Maarel, S.M., de Kok, Y.J.M., Ropers, H.H., Cremers, F.P.M., Pembrey, M. and Malcolm, S. (1995) Further mutations in *Brain 4* (POU3F4) clarify the phenotype in the X-linked deafness DFN3. *Hum. Mol. Genet.*, **4**, 1467–1469.

16 Henikoff, S (1990) Position-effect variegation after 60 years *Trends Genet*, **6**, 422–426
17 Foster, J W Dominguez Steglich M A, Guioli S Kwok C Weller, P A Stevanovic, M, Weissenbach, J Mansour S, Young, I D Goodfellow, P N, Brook, J D and Schafer, A J (1994) Campomelic dysplasia and autosomal sex reversal caused by mutations in an *SRY* related gene *Nature*, **372**, 525–530
18 Wagner, T, Wirth J, Meyer, J, Zabel, B, Held, M, Zimmer J Pasantes, J, Dagna Bricarelli, F, Keutel, J, Hustert, E, Wolf U, Tommerup N, Schempp, W and Scherer, G (1994) Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the *SRY* related gene *SOX9 Cell*, **79**, 1111–1120
19 Fantes, J, Redeker B, Breen, M, Boyle, S, Brown, J, Fletcher, J, Jones, S Bickmore W Fukushima, Y, Mannens M, Danes, S van Heyningen, V and Hanson, I (1995) Aniridia-associated cytogenetic rearrangements suggest that a position effect may cause the mutant phenotype *Hum Mol Genet* **4** 415–422
20 van der Maarel, S M, Scholten, I H J M, Maat-Kievit, J A, Huber, I, de Kok, Y J M, de Wijs I, van de Pol, T J R, van Bokhoven, H den Dunnen, J T, van Ommen G J B, Philippe, C, Monaco, A P, Smeets H J M, Ropers H-H and Cremers, F P M (1995) Yeast artificial chromosome cloning of the Xq13 3–q21 31 region and fine mapping of a deletion associated with choroideremia and non-specific mental retardation *Eur J Hum Genet*, in press
21 Grosveld, F, Blom van Assendelft G Greaves D R and Kollias G (1987) Position independent, high-level expression of the human β globin gene in transgenic mice *Cell*, **51**, 975–985
22 Higgs, D R Wood W G, Jarman, A P, Sharpe J, Lida, J, Pretorius, I-M and Ayyub H (1990) A major positive regulatory region located far upstream of the human α-globin gene locus *Genes Dev*, **4**, 1588–1601
23 Bitner Glindzicz, M, de Kok, Y, Summers, D, Huber, I, Cremers, F P M, Ropers, H-H, Reardon, W, Pembrey, M E and Malcolm, S (1994) Close linkage of a gene for X linked deafness to three microsatellite repeats at Xq21 in radiologically normal and abnormal families *J Med Genet*, **31**, 916–921
24 Reardon, W, Bellman, S Phelps P Pembrey, M and Luxon L M (1993) Neuro otological function in X linked hearing loss a multipedigree assessment and correlation with other clinical parameters *Acta Oto laryngol* **113**, 706–714
25 Ayazi, S (1981) Choroideremia, obesity and congenital deafness *Am J Ophthalmol*, 92, 63–69
26 Nussbaum, R L, Lesko, J G, Lewis, R A, Ledbetter, S A and Ledbetter, D H (1987) Isolation of anonymous DNA sequences from within a submicroscopic X chromosomal deletion in a patient with choroideremia, deafness, and mental retardation *Proc Natl Acad Sci USA*, **84**, 6521–6525
27 Orita, M, Iwahana, H, Kanazawa, H, Hayashi, K and Sekiya, T (1989) Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms *Proc Natl Acad Sci USA*, **86**, 2766–2770
28 Suijkerbuijk R F, van de Veen, A Y, van Echten, J, Buys, C H C M de Jong, B, Oosterhuis, J W, Warburton, D A, Cassiman, J J Schonk D and Geurts van Kessel A (1991) Demonstration of the genuine iso 12p character of the standard marker chromosome of testicular germ cell tumors and identification of further chromosome 12 aberrations by competitive in situ hybridization *Am J Hum Genet*, **48**, 269–273
29 Suijkerbuijk, R F, Looijenga, L, de Jong, B, Oosterhuis, J W, Cassiman, J J and Geurts van Kessel, A (1992) Verification of isochromosome 12p and identification of other chromosome 12 aberrations in gonadal and extragonadal human germ cell tumors by bicolor double fluorescence in situ hybridization *Cancer Genet Cytogenet*, **6**, 8–16
30 Sinke, R J, Suijkerbuijk, R F, Herbergs, J, Janssen, H, Cassiman, J J and Geurts van Kessel, A (1992) Generation of a panel of somatic cell hybrids containing fragments of human chromosome 12p by X Ray irradiation and cell fusion *Genomics*, **12**, 206–213

CHAPTER 6

FINE-MAPPING OF THREE X CHROMOSOMAL BREAKPOINTS ASSOCIATED WITH MENTAL RETARDATION (MR): FURTHER EVIDENCE FOR GENETIC HETEROGENEITY OF X-LINKED MR

Silvère M van der Maarel, Susann Schweiger, Jean-Pierre Fryns, Niels Tommerup, Kristine Kingsley, Daniel Olde Weghuis, Wolfgang Berger, Hugues Roest Crollius, Frans P M Cremers and Hans-Hilger Ropers

Submitted for publication

Abstract

Mapping studies have been reported for 42 families with non-syndromic X-linked mental retardation (XLMR) (1), and have indicated that the X chromosome harbours at least 8 distinct loci for this condition based on the non-overlapping intervals of linkage in these families (2) Moreover, in almost half of the families, the underlying gene defect maps to the proximal short arm of the X chromosome which may be indicative for the presence of one major gene for non-syndromic XLMR in this chromosomal segment In this study, we have employed fluorescence *in situ* hybridization of Yeast Artificial Chromosome (YAC) probes to map the X chromosomal breakpoints in three mentally retarded females carrying balanced X,autosome translocations which had been assigned cytogenetically to the Xp11 band Identification of YACs spanning these breakpoints revealed that all of them map to different X chromosomal sub-bands, i e Xp11 2, Xp11 3, and Xp21 1 Moreover, characterization of the autosomal breakpoints excluded the involvement of autosomal candidate genes, which renders a disruption of an X chromosomal MR-gene the most plausible explanation for the observed phenotype Molecular characterization of these breakpoints should soon reveal which of the relevant genes plays a role in XLMR

Introduction

Various population studies have revealed that in males, the incidence of mental retardation (MR) is significantly higher than in females These studies suggest that X-linked genes account for 20 to 50% of all cases with MR and that between 1 in 300 and 1 in 500 males are affected On the basis of these data it has been estimated that there are at least 7-19 genes on the human X chromosome that play a major role in MR (3-7) To date, almost 150 X-linked conditions have been reported where MR is the only clinical feature or where MR is part of a syndrome (1) Despite the frequency of this condition, little is known about the underlying molecular defects Only a few of the genes involved in specific forms of XLMR could be isolated, including Fragile X syndrome, MASA syndrome (mental retardation, aphasia, shuffling gait and adducted thumbs), and the X-linked mental retardation / a-thalassemia syndrome (ATR-X) (8-11) For the great majority of patients and their families, however, neither counselling nor diagnosis is possible In particular, this holds true for non-syndromic XLMR, i e MR without other clinical features Only recently, two candidate genes for non-syndromic forms of XLMR were described One of these genes, *FMR2*, is associated with a CCG trinucleotide repeat expansion (FRAXE) (12-14), and the second gene, *DXS6673E*, is disrupted by a balanced X,autosome translocation in a mentally retarded female (15) Linkage analysis has shown that at least eight genes are involved in non-syndromic XLMR, and there is evidence for a clustering of linkage intervals in the proximal region of the short arm of the X chromosome (2) This apparent clustering may be due to the presence of one major gene for non syndromic XLMR in this chromosomal segment So far,

however, linkage intervals are mostly wide because they are derived from single families; in the absence of specific clinical features that are characteristic for the different forms of XLMR, linkage data from independent families cannot be pooled. This does not only preclude reliable carrier detection in these families, but virtually rules out the identification of the causative gene defects by positional cloning.

De novo balanced chromosome rearrangements have an incidence of approximately 1:3000 newborns, and in 6% the rearrangement is accompanied by abnormal phenotypes (16). In 50% of these, aberrant clinical features may be causally related to the karyotypic changes, as estimated from the incidence of phenotypic abnormalities in newborns with and without balanced chromosome rearrangements. For X;autosome (X;a) translocations, this proportion may even be higher because truncation of X chromosomal genes will usually result in a functional nullisomy (16-18).

Here, we have identified YACs overlapping three X chromosomal breakpoints in mentally retarded females with balanced X;a translocations. By conventional cytogenetic analysis, all these breakpoints had been assigned to Xp11, but our data show that they map to distinct physical intervals on the proximal Xp. These results argue for the presence of several genes for non-syndromic XLMR on the proximal short arm of the X chromosome and pave the way for their isolation.

Materials and Methods

Patients

Patient TC, 46,X,t(X;20)(p11;q13.3), a 25 year old female, was born as the fifth child of healthy, nonconsanguineous parents. At her birth the father was 31 years and the mother was 29 years old. The first born child of this family, a girl, had died at the age of 3 months from a cardiac malformation. The other siblings, 3 brothers and 1 sister, are normal. Birth weight was 3,1 kg and birth length 49 cm. No medical problems were noted in the perinatal period and the first year of life. Psychomotor development was retarded and expressive language markedly delayed. She could walk without support at the age of 2 years and expressed the first words at the age of 4 years. At the same age generalized epileptic seizures were noted, with good response on anti-epileptic treatment. At age 6 she was integrated in a special school for moderately mentally retarded (IQ_T50, IQ_V46, IQ_P54 - WISC-R scale). After the age of 14 years severe behavioral problems with episodes of aggressive and self destructive behavior became apparent necessitating further diagnostic evaluation. Clinical examination revealed a moderately mentally retarded adolescent female with general hypotrophy, poor muscle development and relative microcephaly (weight 38 kg (third percentile), height 159 cm (50th percentile), head circumference 52 cm (third percentile)). Secondary sexual development was normal and menstrual cycle regular. On her skin disseminated, non telangiectatic, reddish maculae without photosensibility were present. These skin changes remained identical in the following years and

further dermatological examination did not result in a specific diagnosis. Electroencephalography, CT-scan of the brain, routine hematological and metabolic screening all gave normal results. Chromosome studies on peripheral blood lymphocyte cultures and on a fibroblast culture from a skin biopsy at the right forearm revealed an apparently balanced 46,X,t(X;20)(p11.2;q13.3) karyotype after G- and R-banding. The karyotype of both parents was normal.

Patient LMP, 46,X,t(X;17)(p11.3;p13.3) *de novo*. Born at term, Apgar 10/1 - 10/5. She sat at 11 months and her mother reported that she developed normally until age 1 ½ years. She then developed squint, hypotonia, poor balance, and nystagmus, with regression of former abilities. Hospitalisation at age 19 months revealed reduced balance, nystagmus, and truncal and peripheral ataxia. A viral (EBV) encephalitis was initially suspected due to elevated IgM against EBV, but EBV-IgG remained undetectable. No other evidence of immune deficiency has been found, and she has normal IgA, IgM, IgG, and IgG subclasses. CT scan was normal, but EEG was abnormal with paroxystic spikewaves without clinical evidence of seizures. Her development has been retarded, and she only uses a few words at age 4 years. An MR scan at age 3 years was normal with exclusion of lissencephaly. Ophthalmoscopic examination at age 2 years was normal. Chromosome studies revealed an apparently balanced 46,X,t(X;17)(p11.3;p13.3) translocation. Although the autosomal breakpoint colocalizes with the Miller-Dieker syndrome (MDS) region at 17p13.3, the patient does not suffer from lissencephaly. Replication studies revealed

Patient VDBP, 46,X,t(X;15)(p11;q12), a 25 year old female, was born as the second child of healthy, nonconsanguineous parents. At her birth the father was 28 and the mother was 27 years old. Her older brother is normal. Pregnancy and delivery, at 39 weeks, were normal. Birthweight was 2,4 kg and length 46,5 cm. No medical problems were noted in the perinatal period and the first year of life. Psychomotor development was severely retarded from the beginning. She started to walk without support at the age of 2,5 years. After the age of 5 years, she started to express a few, single words. At the age of 11 years she was admitted to an institute for the severely mentally retarded (IQ_T 31, IQ_V 26, IQ_P 32 WISC-R scale).

Severe behavioural problems with episodes of selfdestructive behaviour were noted. Pubertal development was normal: menarche at 12,5 years with regular menstrual cycle; normal secondary sexual characteristics. At the present age of 25 years weight is 62 kg, height 167 cm and head circumference 57 cm. Dysmorphic features are lacking, except a somewhat high and broad forehead and deeply set eyes. Upon neurological examination she was normal. CT-scan of the brain, routine biochemical and hematological screening showed normal results. Routine chromosome analysis on peripheral blood lymphocytes and on a fibroblast culture from a skinbiopsy of the left forearm revealed an apparently balanced Xp/15q translocation with karyotype: 46,X,t(X;15)(p11;q12). Cytogenetic examination of both parents was normal.

Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization (FISH) procedures used were essentially as described elsewhere (19-21) The X chromosome centromere specific alphoid sequence probe pBAMX5 was labeled with digoxigenin-11-dUTP (Boehringer) while the YAC clones were labeled with biotin-14-dATP using a nick-translation kit (Gibco, Life Technologies) 200 ng labelled YAC probe was dissolved in 6 ml of a hybridization solution containing 50% v/v deionized formamide, 10% w/v dextrane sulphate, 2xSSC and 1% v/v Tween-20 at pH7 0 as well as 10 mg Cot-1 DNA (Gibco, Life Technologies) For cosmids, 100 ng labelled probe was dissolved in the presence of 5 mg Cot-1 DNA Prior to hybridization, the probe was denatured at 80°C for 10 minutes, chilled on ice, and incubated for 30 minutes at 37°C allowing preannealing For the pBAMX5 probe, 20 ng of DNA was used in 6 ml of the hybridization solution without competitor DNA Metaphase spreads were prepared using standard procedures After denaturation of the slides, probe incubations were carried out under an 18x18 mm coverslip in a moist chamber for 45 hours

Immunocytochemical detection of the hybridizing probes was achieved as described elsewhere (19-21)

YAC and cosmid clones

For the mapping of the X-chromosomal breakpoints several YAC clones from the Xp11-p21 regions were used YAC clones used for patient TC are part of previously published YAC contigs in Xp11 (22,23) For the X chromosomal breakpoint in patient LMP, we used YACs isolated from a region between *ARAF1* and *NDP* (WB, unpublished data) while YACs for patient VDBP were identified from the integrated X chromosome database (IXDB) (24) YACs from 15q11-q13 encompassing the Prader-Willi and Angelman syndrome region (PWS-AS) were identified from the literature (25) The cosmid clones c11-3 and c18-2 from 20q13 were generously provided by O Steinlein (Bonn, Germany) Yeast cell culturing and DNA isolation was done as described elsewhere (26)

Southern blot analysis

Hybridizations were performed as described (27) Briefly, genomic DNA was digested with restriction enzymes according to the manufacturer's instructions and separated on an agarose gel The DNA was transferred to a membrane (GeneScreen Plus) DNA was hybridized in a solution containing 40mM $NaPO_4$, 7% SDS, 10% polyethylenglycol 6000 and 1mM EDTA supplemented with 10mg/mL herring sperm DNA Probes containing repetitive sequences where hybridized after preannealing with sonicated placental DNA for 18 hrs at 65°C After hybridization, membranes were washed at 65°C in 40mM $NaPO_4$/0 5% SDS for 1 hour

Probe pSyG11-2, containing a human clone encompassing the promoter and the first exon of *SYN-1*, and probe pCMVSyIb containing the bovine cDNA for *SYN-1*, were generously provided by G Thiel (Cologne, Germany) The pTIMP probe (3 9X) is a 3 9 kb genomic *Xba*I fragment containing the *TIMP* gene (28) The

human properdin probe 5/6 is a genomic 0 8 kb *Bam*HI/*Hind*III fragment containing part of the coding sequences of *PFC* (29,30)

Results

Patient TC, 46,X,t(X;20)(p11;q13.3)

YACs from previously established YAC contigs in Xp11 (22,23) were hybridized *in situ* to metaphase chromosomes of the patient Initially, YACs were chosen from a region between *OATL1* and *NDP* since this segment of the X chromosome is well characterized and displays a large number of candidate genes for XLMR Moreover, three genes containing a conserved zinc finger motif (ZFP genes) have been assigned to this region, i e *ZNF21*, *ZNF41*, and *ZNF81* (22,23,31-34) ZFP genes are known to cluster around telomeres and fragile sites, chromosomal segments that seem to correlate with translocations and deletions associated with clinical disorders (32,35-37) Also, linkage analyses suggest the presence of one or several XLMR genes in the *ALAS2*-DXS1003 interval which partially overlaps the above mentioned segment (1,2)

By using *in situ* hybridization analysis, YAC clone C1022 was shown to span the X chromosomal breakpoint in this patient (Fig 1a) This result was confirmed by the hybridization of YAC clones C1228 and C0874 which are known to map distal and proximal to YAC C1022, respectively As expected, C1228 only hybridized to the der(20) while C0874 hybridized only to the der(X) chromosome (data not shown) To rule out the involvement of synapsin I (*SYN-1*) a major component of synaptic vesicles (38,39), we isolated cosmids from an X chromosome specific cosmid library, LL0XNC01 "U", representing a region that includes the genes for properdin P factor (*PFC*), *SYN-1*, and tissue inhibitor of metalloproteinase (*TIMP*) (22) FISH analysis revealed that all cosmids were located distal to the breakpoint (data not shown) Also, hybridization of these cosmids to a Southern blot containing DNA from the patient and a normal female failed to detect novel restriction fragments (Fig 2a) These results rule out a breakpoint in or very close to *SYN-1*

The autosomal breakpoint have been mapped cytogenetically to 20q13 3 To this chromosomal segment also the nicotinic acetylcholine receptor α4 subunit (*CHNRA4*) has been mapped, and mutations in *CHRNA4* have been found in a family with benign familial neonatal convulsions (BFNC) and a family with autosomal dominant nocturnal frontal lobe epilepsy ADNFLE (40,41) Since this patient also exhibited epileptic seazures during the second and third year of life we hybridized *in situ* cosmid c11-3 and cosmid c18-2 that are part of a 200kb cosmid contig containing the *CHNRA4* gene to metaphase chromosomes of the patient Both cosmids hybridize to the derivative X chromosome indicating a proximal localization of this breakpoint with respect to the ADFNLE and BFNC loci (data not shown)

Patient LMP, 46,X,t(X;17)(p11.3;p13.3)

Several YACs from a YAC contig spanning the Xp11 3 region between ARAF1 and MAOA were hybridized *in situ* to metaphase chromosomes of the patient With one of these, YAC CEPHy904E11896, split hybridization signals were observed, albeit only in a minority of metaphases and indicating that this YAC spans the breakpoint (Fig 1b) Moreover, the hybridization results obtained with several neighboring and partially overlapping YACs confirmed this result In this way, the breakpoint could be mapped to the centromeric end of YAC CEPHy904E11896 The 17p13 3 breakpoint has been shown to reside within the Miller-Dieker (MDS) syndrome deletion region, but a previously cloned chimearic lissencephaly (*LIS1*) gene (42-44), is not disrupted by the translocation (N T , personal communication)

Patient VDBP, 46,X,t(X;15)(p11;q12)

YACs used to map the breakpoint in patient LMP were initially hybridized in situ to metaphase chromosomes of patient VDBP All YACs mapped proximally to the breakpoint (data not shown) Subsequently, several YACs from the IXDB YAC contig were hybridized to metaphase chromosomes of the patient Two partially overlapping YAC clones, ICRFy900H0893, and ICRFy900C0413 were identified that recognize the breakpoint (Fig 1c) These YAC clones define the distal border of a YAC contig spanning the Xp11 4-p21 1 region Therefore, at the distal side of these YACs, no immediately adjacent YACs were available to confirm this result However, a YAC located proximal to these breakpoint spanning YACs, ICRFy900E0501, remains on the derivative X chromosome, while most proximal YAC of the nearest distal YAC contig, clone ICRFy900E0101, hybridizes only to the normal X chromosome and the derivative chromosome 15 (data not shown)

The autosomal breakpoint has cytogenetically been mapped to 15q12 and may therefore coincide with the PWS-AS region (45) To map this breakpoint with respect to the PWS-AS region, we hybridized *in situ* several YACs spanning this region (CEPHy904 clones H1111, B1171, B05254, A01264, A12307, F06326, D01495, and St Louis clones A124A3, and A156E1) (46) All YACs hybridized to the derivative X chromosome which indicates that the breakpoint on chromosome 15 is located proximal to the PWS-AS region (data not shown)

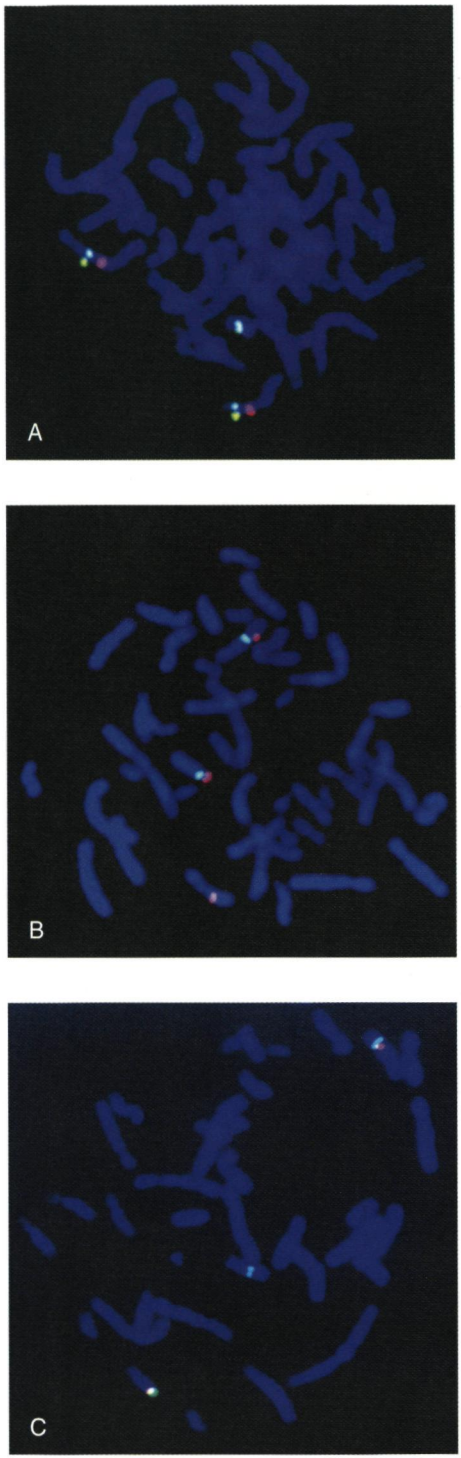


Figure 1
Fluorescence *in situ* hybridization of (a) YAC C1022 to metaphase chromosomes of patient TC, (b) YAC CEPHy904E11896 to metaphase chromosomes of patient LMP, and (c) YAC ICRFy900H0893 to metaphase chromosomes of patient VDBP. All YACs span the breakpoints since their hybridization signals (red) are seen on the normal X chromosome, and the derivative chromosomes. The X centromere is marked in green.

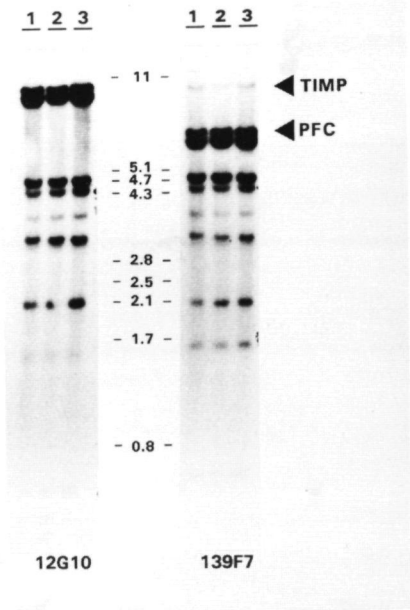
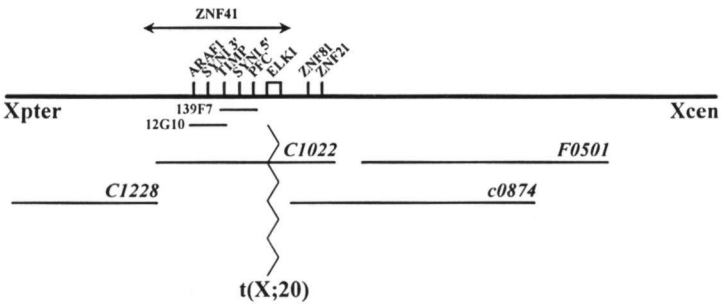


Figure 2
Characterization of the X chromosomal breakpoint in patient TC.
a: Southern blot analysis of the cosmids12G10 and 139F7 to *EcoRI* digested DNAof a female control (lane 1), a male control (lane 2), and patient TC (lane 3). Genomic fragments carrying *TIMP* and *PFC* are marked by arrows.



b:Molecular analysis of the region spanning the X chromosomal breakpoint in patient TC. The upper line represents a segment of the Xp11.23-p11.3 region containing the genes *ARAF1*, *SYN-1*, *TIMP*, *ELK1*, *ZNF41*, *ZNF21*, and *ZNF81*. The localization of *ZNF41* is not completely clear as indicated with the double arrowhead. *ELK1* is located proximally or distally to the breakpoint. The two small lines underneath represent the cosmids from the X chromosome specific cosmid library LL0XNC01 "U" used for Southern blot analysis. The horizontal bars with the italic numbers represent the YAC clones from this region that are used for fluorescence *in situ* hybridization. The localization of the breakpoint is indicated with an arrow.

Discussion

About fifty percent of all disease phenotypes found in patients with balanced chromosome aberrations may be causally related to the karyotypic changes. Balanced translocations and inversions involving autosomes will give rise to disease phenotypes if they hit a gene for which two functional gene copies are required, so that disruption or down regulation of one allele will result in haploinsufficiency (18). Disease phenotypes may also arise when the rearrangement results in dominant mutations (47), if the other allele happens to be mutated (unmasking of heterozygosity) (48), or if there is only one functional gene copy per cell so that its inactivation will result in a functional nullisomy, as for imprinted (49-52) or X and Y chromosomal genes. Therefore, in patients with balanced X;autosome translocations, disease phenotypes will be more frequent than in other balanced translocations. This does not only hold for males but also for females where disruption of X chromosomal genes will generally lead to functional nullisomy, too, because of preferential inactivation of the normal X chromosome (17,53). This renders balanced X chromosomal rearrangements particularly valuable as tools for the identification of genes that play a role in disease. Indeed, many of the disease genes that have been identified by positional cloning strategies are X chromosomal, and X chromosomal rearrangements were instrumental for their isolation (reviewed in 54). For the identification of genes involved in XLMR, the molecular characterization of X chromosomal rearrangements associated with MR may be almost without alternative due to the heterogeneity of the disorder and the relatively wide linkage intervals in families with XLMR. Here, employing YACs, we have mapped three X chromosomal breakpoints that are associated with MR to the Xp11-p21 region.

The breakpoint of patient TC in Xp11.2 is located in a well-defined segment. Several genes in this region may be considered as candidate genes for XLMR like *SYN-1*, a major component of the synaptic vesicles, and the putative transcriptional regulators, *ZNF21*, *ZNF41* and *ZNF81* (22,23,31-34). Direct involvement of *SYN-1* in this translocation was ruled out by FISH and Southern blot experiments involving a cosmid contig that spans the region from *PFC* to *ARAF1* (Fig.2a). However, we cannot exclude a disruption of the *SYN-1* transcription unit as a result of the X;autosome translocation since the 5' end of *SYN-1* is orientated towards the breakpoint. These results position the breakpoint to a small segment of approximately 200 kb proximal to *PFC* and distal to YAC C0874 (Fig 2b). This region also contains *ELK1* and possibly *ZNF41*, while *ZNF21* and *ZNF81* map 200-300 kb proximal to *ELK1*. Their involvement in the translocation cannot be excluded. ZNP genes encode putative transcriptional regulators with key regulatory functions during development (55). So far, ZNP's were shown to be involved in tumorgenesis in Wilms' tumor, and in limb and craniofacial development in the Greig cephalopolysyndactyly syndrome (56-58). The respective genes, *WT1* and *GLI3* together with the three ZNP genes mentioned above, belong to the Krüppel gene family. *ELK1* codes for a member of the v-*ets* oncogene superfamily. It has been suggested that proteins of this superfamily

have DNA binding capacities. This gene appears to be transcribed in lung and testis which would argue against its direct involvement in MR (59).

The autosomal breakpoint of this translocation was cytogenetically mapped to 20q13, a chromosomal segment to which three autosomal dominant types of epilepsy, ADNFLE, BFNC, and low voltage EEG (EEGV1), have also been mapped. Although mutations in the *CHRNA4* gene in ADFNLE and BFNC have demonstrated genetic allelism for both disorders, the molecular cause for EEGV1 has not been elucidated yet (40,41,60). The presence of generalized epileptic seizures during the fourth year of life in this patient prompted us to assess the involvement of the *CHRNA4* gene in the phenotype of the patient. The observation that two cosmids, representing both ends of a 200kb interval of genomic DNA around the *CHRNA4* gene, both map distal to the breakpoint on chromosome 20 suggests that *CHRNA4* gene expression is probably not impaired in this patient.

The X chromosomal breakpoint in patient LMP maps to Xp11.3 between DXS1055 and DXS1708, and there are no obvious candidate genes mapped to this region. We estimate that the size of the breakpoint region is smaller than 300kb based on the sizes and FISH hybridization results of other YAC clones from this contig. The autosomal breakpoint has been mapped in the vicinity of the MDS region but its involvement in the clinical phenotype seems unlikely since the patient does not show any dysmorphic signs characteristic for MDS.

The X chromosomal breakpoint in patient VDBP had been cytogenetically mapped to Xp11. However, our in situ hybridizations indicate a more distal localization in Xp21.1 near the McLeod gene (*XK*) locus (61). Two partially overlapping YAC clones recognize the breakpoint and, the phenotype of the patient argues against involvement of the *XK* gene itself. Several patients have been described with deletions that include the X chromosomal breakpoint locus (61) and references therein). Since these patients are not mentally retarded, the MR in patient VDBP may be due to a position effect, or to disruption of a gene on chromosome 15. The autosomal breakpoint has been assigned to 15q12 to which also the genes for PWS and AS have been mapped (reviewed in(45). PWS and AS are MR syndromes for which imprinting has been implicated. Therefore, disruption of the PWS-AS region by a translocation should give rise to a disease if the breakpoint involves the only functional copy of these genes. Indeed, a translocation has been identified in a patient with PWS directly disrupting the maternally imprinted small nuclear ribonucleoprotein (*SNRPN*) transcript (62). To rule out the possibility that the PWS or AS genes are disrupted in patient VDBP, too, we hybridized several YACs from the PWS and AS region to metaphase spreads of the patient. All YACs, including those that define the proximal limit of deletions that are associated with PWS or AS (63) are located distal to the breakpoint on chromosome 15. Therefore, involvement of PWS-AS genes on chromosome 15 is unlikely.

Although we have excluded direct disruption of the respective obvious autosomal candidate genes in these patients, we cannot rule out indirect effects on their transcriptional activity. All these loci are translocated to the derivative X

chromosome carrying the X Inactivation Center (XIC) (reviews in 64,65), and spreading of X inactivation into the autosomal segment in a small subset of cells with incomplete inactivation of the normal X chromosome may influence proper expression of these genes (17,53) Alternatively, these translocations may juxtapose heterochromatic sequences near the autosomal loci thereby down-regulating transcription of these autosomal genes This phenomenon, position effect variegation (PEV), was first described in *D melanogaster* (66), and may explain some rare observations in humans (reviewed in 67)

The most straight-forward explanation for the aberrant clinical phenotypes in these patients is that these rearrangements result in a functional nullisomy of X chromosomal genes Previous linkage analyses have revealed a clustering of XLMR loci to the proximal short arm of the X chromosome (1,2) These linkage data may indicate that there is a common, particularly important XLMR gene in this region, or alternatively, that several XLMR genes may be clustered in this chromosomal segment Since the breakpoints described here do not coincide, but map to distinct subbands in proximal Xp, our results suggest, but do not prove, the presence of several XLMR genes on the proximal short arm of the X chromosome The detailed molecular characterization of the X chromosomal rearrangements described here, should provide us important clues to their identification

Acknowledgements

The autors are indebted to Mrs S D van der Velde-Visser and E Boender-van Rossum for expert technical assistance We thank Mrs V Suckow for her contribution to this project, Mrs E van de Vosse for supplying cosmids from YAC Screening Center Leiden Probes pSyG11-2 and pCMVSyIb, and the cosmids c11-3 and c18-2 were gifts from G Thiel and O Steinlein, respectively The research of F P M C has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences This work was supported by The Netherlands Organization of Scientific Research (NWO grant 960-10-809) and the Dutch Praeventiefonds (grant 28-2447) The chromosome-specific cosmid library LL0XNC01 used in this work was constructed at the Biology & Biotechnology Research Program, Lawrence Livermore National Laboratory, Livermore, CA 94550 under auspices of the National Gene Library Project sponsored by the U S Department of Energy

References

1 Lubs, H A , Chiurazzi, P , Arena, J F , Schwartz, C , Tranebjaerg, L , and Neri, G (1996) XLMR genes update 1996 *Am J Med Genet* , **64**, 147 157

2 Gedeon, A K , Donnelly, A J , Mulley, J C , Kerr, B , and Turner, G (1996) How many X-linked genes for non-specific mental retardation (MRX) are there? *Am J Med Genet* , **64**, 158-162

3 Herbst, D S and Miller, J R (1980) Nonspecific X-linked mental retardation II the frequency in British Columbia *Am J Med Genet* , **7**, 461-469

4 Opitz, J M (1986) Editorial comment on the gates of hell and a most unusual gene *Am J Med Genet* , **23**, 1-10

5 Opitz, J M (1987) Erratum *Am J Med Genet* , **26**, 37

6 Glass, I A (1991) X linked mental retardation *J Med Genet* , **28**, 361-371

7 Kerr, B , Turner, G , Mulley, J , Gedeon, A , and Partington, M (1991) Non-specific X-linked mental retardation *J Med Genet* , **28**, 378-382

8 Verkerk, A J M H , Pieretti, M , Sutcliffe, J S , Fu, Y , Kuhl, D P A , Pizzuti, A , Reiner, O , Richards, S , Victoria, M F , Zhang, F , Eussen, B E , van Ommen, G B , Blonden, L A J , Riggins, G J , Chastain, J L , Kunst, C B , Galjaard, H , Caskey, C T , Nelson, D L , Oostra, B A , and Warren, S T (1991) Identification of a gene (*FMR-1*) containing a CGG repeat coincident with a fragile X breakpoint cluster region exhibiting length variation in fragile X syndrome *Cell*, **65**, 905-914

9 Jouet, M , Rosenthal, A , Armstrong, G MacFarlane J , Stevenson, R , Paterson J , Metzenberg, A , Ionasescu, V , Temple, K , and Kenwrick, S (1994) X-linked spastic paraplegia (SPG1), MASA syndrome and X-linked hydrocephalus result from mutations in the *L1* gene *Nature Genet* , **7**, 402-407

10 Vits, L , van Camp, G , Coucke, P , Fransen, E , De Boulle, K , Reyniers, E , Korn, B , Poustka, A , Wilson, G , Schrander-Stumpel, C , Winter, R M , Schwartz, C , and Willems, P J (1994) MASA syndrome is due to mutations in the neural cell adhesion gene *L1CAM Nature Genet* , **7**, 408-413

11 Gibbons, R J , Picketts, D J , Villard, L , and Higgs, D R (1995) Mutations in a putative global transcriptional regulator cause X-linked mental retardation with a-thalassemia (ATR-X syndrome) *Cell*, **80**, 837-845

12 Chakrabarti, L , Knight, S J L , Flannery, A V , and Davies, K E (1996) A candidate gene for mild mental handicap at the FRAXE fragile site *Hum Mol Genet* , **5**, 275-282

13 Gecz, J , Gedeon, A K , Sutherland, G R , and Mulley, J C (1996) Identification of the gene *FMR2*, associated with *FRAXE* mental retardation *Nature Genet* , **13**, 105-108

14 Gu, Y , Shen, Y , Gibbs, R A , and Nelson, D L (1996) Identification of *FMR2*, a novel gene associated with the *FRAXE* CCG repeat and CpG island *Nature Genet* , **13**, 109-113

15 van der Maarel, S M , Scholten, I H J M , Huber, I , Philippe, C , Suijkerbuijk, R F , Gilgenkrantz, S , Kere, J , Cremers, F P M , and Ropers, H -H (1996) Cloning and characterization of *DXS6673E*, a candidate gene for X-linked mental retardation in Xq13 1 *Hum Mol Genet* , **5**, 887-897

16 Warburton, D (1991) De novo balanced chromosome rearrangements and extra marker chromosomes identified at prenatal diagnosis clinical significance and distribution of breakpoints *Am J Hum Genet*, **49**, 995-1013

17 Schmidt, M and Du Sart, D (1992) Functional disomies of the X chromosome influence the cell selection and hence the X inactivation pattern in females with balanced X-autosome translocations a review of 122 cases *Am J Med Genet*, **42**, 161-169

18 Tommerup, N (1993) Mendelian cytogenetics Chromosome rearrangements associated with mendelian disorders *J Med Genet*, **30**, 713-727

19 Suijkerbuijk, R F , van de Veen, A Y , van Echten, J , Buys, C H C M , de Jong, B , Oosterhuis, J W Warburton, D A , Cassiman, J J , Schonk, D , and Geurts van Kessel, A (1991) Demonstration of the genuine iso-12p character of the standard marker chromosome of testicular germ cell tumors and identification of further chromosome 12 aberrations by competitive in situ hybridization *Am J Hum Genet*, **48**, 269-273

20 Smke, R J , Suijkerbuijk, R F , Herbergs, J , Janssen, H , Cassiman, J J , and Geurts van Kessel, A (1992) Generation of a panel of somatic cell hybrids containing fragments of human chromosome 12p by X-Ray irradiation and cell fusion *Genomics*, **12**, 206-213

21 Suijkerbuijk, R F , Looijenga, L , de Jong, B , Oosterhuis, J W , Cassiman, J J , and Geurts van Kessel, A (1992) Verification of isochromosome 12p and identification of other chromosome 12 aberrations in gonadal and extragonadal human germ cell tumors by bicolor double fluorescence in situ hybridization *Cancer Genet Cytogenetic*, **6**, 8-16

22 Derry, J M J and Barnard, P J (1992) Physical linkage of the A-*raf*-1, properdin, synapsin I, and TIMP genes on the human and mouse X chromosomes *Genomics*, **12**, 632-638

23 Knight, J C , Grimaldi, G , Thiesen, H , Bech-Hansen, N T , Fletcher, C D M , and Coleman, M P (1994) Clustered organization of *Kruppel* zinc-finger genes at Xp11 23, flanking a translocation breakpoint at OATL1 a physical map with locus assignments for ZNF21, ZNF41, ZNF81, and ELK1 *Genomics*, **21**, 180-187

24 Roest Crollius, H , Ross, M T , Grigoriev, A , Knights, C J , Holloway, E , Misfud, J , Li, K , Playford, M , Gregory, S G , Humphray, S J , Coffey, A J , See, C G , Marsh, S , Vatcheva, R , Kumlien, J , Labella, T , Lam, V , Rak, K H , Todd, K , Mott, R , Graeser, D , Rappold, G , Zehetner, G , Poustka, A , Bentley, D R , Monaco, A P , and Lehrach, H (1996) An integrated YAC map of the human X chromosome *Genome Research*, **6**, 943-955

25 Mutirangura, A , Jayakumar, A , Sutcliffe, J S , Nakao, M , McKinney, M J , Buiting, K , Horsthemke, B , Beaudet, A L , Chinault, A C , and Ledbetter, D H (1993) A complete YAC contig of the Prader-Willi/Angelman chromosome region (15q11-q13) and refined localization of the SNRPN gene *Genomics*, **18**, 546-552

26 Green, E D and Olson, M V (1990) Systematic screening of yeast artificial-chromosome libraries by use of the polymerase chain reaction *Proc Natl Acad Sci USA*, **87**, 1213-1217

27 Huber, I , Bitner-Glindzicz, M , de Kok, Y J M , van der Maarel, S M , Ishikawa-Brush, Y , Monaco, A P , Robinson, D , Malcolm, S , Pembrey, M E , Brunner, H G , Cremers, F P M , and Ropers, H (1994) X-linked mixed deafness (DFN3) cloning and characterization of the critical region allows the identification of novel microdeletions *Hum Mol Genet*, **3**, 1151-1154

28 Kidd, K K , Bowcock, A M , Schmidtke, J , Track, R K , Riccuiti, F , Hutchings, G , Bale, A., Pearson, P , and Willard, H F (1989) Report of the cDNA committee and catalogs of cloned and mapped genes and DNA polymorphisms *Cytogenet Cell Genet* , **51**, 622-947

29 Nolan, K F , Schwaeble, W , Kaluz, S , Dierich, M P , and Reid, K B M (1991) Molecular cloning of the cDNA coding for properdin, a positive regulator of the alternative pathway of human complement. *Eur J Immunol* , **21**, 771-776

30 Nolan, K F., Kaluz, S , Higgins, J M G , Goundis, D , and Reid, K B.M (1992) Characterization of the human properdin gene *Biochem J* , **287**, 291-297

31. Franzè, A , Archidiacono, N., Rocchi, M , Marino, M , and Grimaldi, G. (1991) Isolation and expression analysis of a human zinc finger gene (ZNF41) located on the short arm of the X chromosome. *Genomics*, 7, 728-736.

32 Huebner, K., Druck, T , Croce, C M., and Thiesen, H (1991) Twenty-seven non-overlapping zinc finger cDNAs from human T cells map to nine different chromosomes with apparent clustering. *Am J Hum Genet* , **48**, 726-740

33. Marino, M , Archidiacono, N , Franzè, A , Rosati, M., Rocchi, M , Ballabio, A., and Grimaldi, G (1993) A novel X-linked member of the human zinc finger protein gene family: isolation, mapping, and expression *Mamm Genome*, **4**, 252-257

34. Hagemann, T , Surosky, R , Monaco, A P , Lehrach, H , Rosen, F S , and Kwan, S (1994) Physical mapping in a YAC contig of 11 markers on the human X chromosome in Xp11 23 *Genomics*, **21**, 262-265

35 Seite, P., Huebner, K , Rousseau-Merck, M F , Berger, R., and Thiesen, H (1991) Two genes encoding zinc finger proteins, ZNF12 (KOX3) and ZNF26 (KOX20) map to chromosome 7p22-p21 and 12q24-33, respectively. *Hum Genet.*, **86**, 585-590.

36. Aubry, M , Demczuk, S , Desmaze, C , Aikem, M , Aurias, A , Julien, J -P., and Rouleau, G.A. (1993) Isolation of a zinc finger gene consistently deleted in DiGeorge syndrome. *Hum Mol Genet* , **2**, 1583-1587.

37. Tommerup, N , Aagaard, L , Lund, C.L., Boel, E., Baxendale, S , Bates, G.P., Lehrach, H , and Vissing, H. (1993) A zinc-finger gene for the Wolf-Hirschhorn (4p-) syndrome. *Hum Mol Genet* , **2**, 1571-1575.

38. Kilimann, M W and DeGennaro, L J (1985) Molecular cloning of cDNAs for the nerve-cell specific phosphoprotein, synapsin I. *EMBO J* , **4**, 1997-2002

39 Sudhof, T.C. (1990) The structure of the human synapsin I gene and protein. *J Biol Chem* , **265**, 7849-7852

40 Beck, C., Moulard, B., Steinlein, O , Guipponi, M , Valle, L., Montpied, P., Baldy-Moulnier, M , and Malafosse, A. (1994) A nonsense mutation in the a4 subunit of the nicotinic acetylcholine receptor (CHRNA4) cosegregates with 20q-linked benign neonatal familial convulsions (EBN1) *Neurobiol Dis.*, **1**, 95-99.

41. Steinlein, O K , Mulley, J.C., Propping, P., Wallace, R H., Phillips, H A , Sutherland, G R., Scheffer, I E , and Berkovic, S F. (1995) A missense mutation in the neuronal nicotinic acetylcholine receptor alpha 4 subunit is associated with autosomal dominant nocturnal frontal lobe epilepsy. *Nature Genet* , **11**, 201-203

42 Reiner, O , Carrozzo, R., Shen, Y , Wehnert, M , Faustinella, F., Dobyns, W B., Caskey, C.T., and Ledbetter, D H (1993) Isolation of a Miller-Dieker lissencephaly gene containing G protein beta-subunit-like repeats. *Nature*, **364**, 717-721

43 Chong, S S , Pack, S , Tanigami, A , Carrozzo, R , Dobyns, W.B and Ledbetter, D H. (1995) Systematic deletion analysis of MDS and ILS patients excludes a candidate gene delineates the lissencephaly gene locus *Am J Hum Genet.*, **57**, A34-(170) (Abstract)

44 Chong, S S , Tanigami, A., Roschke, A V , and Ledbetter, D H (1996) 14-3-3-Î has no homology to *LIS1* and lies telomeric to it on chromosome 17p13 3 outside the Miller-Dieker syndrome chromosome region *Genome Research*, **6**, 735-741.

45 Nicholls, R.D. (1993) Genomic imprinting and candidate genes in the Prader-Willi and Angelman syndromes [published erratum appears in Curr Opin Genet Dev 1993 Oct,3(5):802]. *Curr Opin Genet Dev* , **3**, 445-456.

46 Kuwano, A , Mutirangura, A., Dittrich, B., Buiting, K., Horsthemke, B , Saitoh, S, Niikawa, N., Ledbetter, S A , Greenberg, F., Chinault, A.C , and et al. (1992) Molecular dissection of the Prader-Willi/Angelman syndrome region (15q11-13) by YAC cloning and FISH analysis [published erratum appears in Hum Mol Genet 1992 Dec;1(9) 784] *Hum Mol Genet.*, **1**, 417-425

47 Park, M (1995) In Scriver, C.R., Beaudet, A.L , Sly, W S. and Valle, D. (ed), The metabolic and molecular bases of inherited disease McGraw-Hill,Inc, New York, pp. 589.-611.

48 Bühler, E.M (1983) Unmasking of heterozygosity by inherited balanced translocations. Implications for prenatal diagnosis and gene mapping *Ann Genet-Paris*, **26**, 133-137.

49 Pembrey, M., Fennell, S J., Van den Berghe, J , Fitchett, M , Summers, D , Butler, L , Clarke, C , Griffiths, M , Thompson, E , Super, M , and Baraitser, M (1989) The association of Angelman's syndrome with deletions within 15q11-13 *J Med Genet* , **26**, 73-77

50. Butler, M.G. (1990) Prader-Willi syndrome. current understanding of cause and diagnosis. *Am. J Med Genet.*, **35**, 319-332.

51 Norman, A.M., Read, A.P., Clayton-Smith, J., Andrews, T., and Donnai, D (1992) Recurrent Wiedemann-Beckwith syndrome with inversion of chromosome (11)(p11.2p15.5) *Am J Med Genet* , **42**, 638-641.

52 Mannens, M., Hoovers, J.M.N., Redeker, E., Verjaal, M., Feinberg, A P., Little, P., Boavida, M , Coad, N., Steenman, M , Bliek, J., Niikawa, N , Tonoki, H., Nakamura, Y., de Boer, E G., Slater, R M , John, R , Cowell, J K , Junien, C., Henry, I., Tommerup, N., Weksberg, R., Pueschel, S.M , Leschot, N J., and Westerveld, A. (1994) Parental imprinting of human chromosome region 11p15.3-pter involved in the Beckwith-Wiedemann syndrome and various human neoplasia. *Eur. J Hum. Genet* , **2**, 3-23.

53 Mattei, M.G., Mattei, J F., Ayme, S , and Giraud, F (1982) X-autosome translocations. cytogenetic characteristics and their consequences *Hum Genet.*, **61**, 295-309.

54 Collins, F S. (1995) Positional cloning moves from perditional to traditional *Nature Genet* , **9**, 347-350.

55. El-Baradi, T and Pieler, T (1991) Zinc finger proteins. what we know and what we would like to know *Mech Dev* , **35**, 155-169

56 Call, K M., Glaser, T., Ito, C Y , Buckler, A.J., Pelletier, J., Haber, D A , Rose, E.A , Kral, A., Yeger, H , Lewis, W.H., Jones, C , and Housman, D.E. (1990) Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus *Cell*, **60**, 509-520.

57. Gessler, M , Poustka, A., Cavenee, W , Neve, R.L , Orkin, S H , and Bruns, G.A.P. (1990) Homozygous deletion in Wilms' tumours of a zinc-finger gene identified by chromosome jumping *Nature*, **343**, 774-778.

58 Vortkamp, A , Gessler, M , and Grzeschik, K (1991) GLI3 zinc-finger gene interrupted by translocations in Greig syndrome families *Nature*, **352**, 539-540

59. Rao, V.N., Huebner, K., Isobe, M., ar-Rushdi, A , Croce, C.M , and Reddy, E.S P (1989) *elk*, Tissue-specific *ets*-related genes on chromosomes X and 14 near translocation breakpoints. *Science*, **244**, 66-70

60 Steinlein, O., Anokhin, A , Yping, M , Schalt, E , and Vogel, F. (1992) Localization of a gene for the human low-voltage EEG on 20q and genetic heterogeneity *Genomics*, **12**, 69-73.

61 Ho, M., Chelly, J , Carter, N , Danek, A., Crocker, P , and Monaco, A P (1994) Isolation of the gene for McLeod syndrome that encodes a novel membrane transport protein *Cell*, **77**, 869-880.

62. Sun, Y., Nicholls, R D., Butler, M.G., Saitoh, S., Hamline, B.E , and Palmer, C.G. (1996) Breakage in the *SNRPN* locus in a balanced 46,XY,t(15;19) Prader-Willi syndrome patient. *Hum Mol Genet* , **5**, 517-524.

63 Christian, S.L., Robinson, W P , Huang, B , Mutirangura, A , Line, M R., Nakao, M., Surti, U , Chakravarti, A , and Ledbetter, D H (1995) Molecular characterization of two proximal deletion breakpoint regions in both Prader-Willi and Angelman syndrome patients. *Am J Hum Genet* , **57**, 40-48

64 Migeon, B R. (1994) X-chromosome inactivation. molecular mechanisms and genetic consequences. *Trends Genet.*, **10**, 230-235.

65 Rastan, S (1994) X chromosome inactivation and the *Xist* gene *Curr Opin Genet. Dev* , **4**, 292-297

66. Henikoff, S (1990) Position-effect variegation after 60 years. *Trends Genet* , **6**, 422-426.

67. Bedell, M.A., Jenkins, N.A , and Copeland, N.G. (1996) Good genes in bad neighbourhoods. *Nature Genet.*, **12**, 229-232

CHAPTER 7

CLONING AND CHARACTERIZATION OF *DXS6673E*, A CANDIDATE GENE FOR X-LINKED MENTAL RETARDATION IN XQ13.1

van der Maarel, S M , Scholten, I H J M , Huber, I , Philippe, C , Suijkerbuijk, R F , Gilgenkrantz, S , Kere, J , Cremers, F P M , and Ropers, H -H
Hum Mol Genet , 5, 887-897 (1996)

ARTICLE

Cloning and characterization of *DXS6673E*, a candidate gene for X-linked mental retardation in Xq13.1

Silvère M. van der Maarel[1,2,*], Inge H. J. M. Scholten[1], Irene Huber[1], Christophe Philippe[3], Ron F. Suijkerbuijk[1], Simone Gilgenkrantz[4], Juha Kere[5,+], Frans P. M. Cremers[1] and Hans-Hilger Ropers[1,2]

[1]Department of Human Genetics, University Hospital Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands, [2]Max-Planck-Institut fur Molekulare Genetik, Ihnestraße 73, D-14195 Berlin, Germany, [3]The Wellcome Trust Centre for Human Genetics, Oxford, UK, [4]Centre Hospitale Universitaire de Nancy, Nancy, France and [5]Washington University School of Medicine, St Louis, USA

Received February 20 1996 Revised and Accepted April 17 1996

In several families with non-specific X-linked mental retardation (XLMR) linkage analyses have assigned the underlying gene defect to the pericentromeric region of the X chromosome, but none of these genes have been isolated so far. Here, we report on the cloning and characterization of a novel gene, *DXS6673E*, that maps to Xq13.1, is subject to X-inactivation and is disrupted in the 5′ untranslated region by a balanced X;13 translocation in a mentally retarded female. The *DXS6673E* gene is highly conserved among vertebrates and its expression is most abundant in brain. It encodes a hydrophilic protein of 1358 amino acids (aa) that does not show sequence homology to other known proteins. A segment of this protein consisting of neutral and hydrophobic aa with a proline residue in every second position may represent a transmembrane domain. Almost complete sequence identity was found between the 3′ end of the *DXS6673E* gene and two expressed sequence tags (ESTs) and between the 5′ end of the *DXS6673E* gene and a third EST. Moreover, weaker sequence similarity was observed between coding regions and two other ESTs.

INTRODUCTION

It has long been recognized that the X chromosome contributes disproportionally to the total number of loci that are involved in mental retardation (MR). Population studies have revealed that the prevalence of X linked mental retardation (XLMR) in males varies from 1 300 to about 1 500 and have suggested that X linked genes account for approximately 20–50% of all cases with MR. On the basis of data it has been estimated that at least seven to 19 X chromosomal genes play a major part in MR (1–5). Recently, several gene defects underlying specific forms of XLMR were identified, such as the fragile X syndrome (FraXA), MASA syndrome (mental retardation, aphasia, shuffling gait and adducted thumbs) and mental retardation/α-thalassaemia (ATR-X) syndrome (6–9). In contrast to these disorders with specific recognizable phenotypes or karyotypes, there are many forms of non specific XLMR without additional clinical features. So far, the genetic heterogeneity of non-specific XLMR has prevented its molecular elucidation and for the majority of cases with this condition, neither carrier detection nor early diagnosis is possible.

Linkage analyses in individual families have revealed that there are at least six genes on the X chromosome that play a part in non specific XLMR (10,11). For all of these genes however linkage intervals are still too wide to allow reliable genetic counselling and the identification of causative gene defects by positional cloning. Cytogenetically recognizable chromosomal rearrangements such as X autosome translocations have greatly facilitated the positional cloning of disease-associated gene defects (12). Balanced X-autosome translocations and inversions have also been described in several patients with MR (13–16) and the characterization of the relevant breakpoint regions on the X chromosome is therefore a promising strategy to identify candidate genes for XLMR.

Here, we report on the identification and characterization of an X chromosomal gene, *DXS6673E*, that is disrupted by a balanced X;13 translocation involving the Xq13.1 band in a mentally retarded female. Apart from mental retardation, scoliosis and spotty abdominal hypopigmentation are the only other conspicuous clinical features in this patient (15,17).

RESULTS

Identification of a YAC that recognizes the X chromosomal breakpoint

A hybrid cell line containing the derivative chromosome 13 as its only human X-chromosomal component (17) was used to

*To whom correspondence should be addressed at Max Planck Institut fur Molekulare Genetik, Ihnestraße 73, D-14195 Berlin Dahlem, Germany
+Present address: Department of Medical Genetics, University of Helsinki, Helsinki, Finland

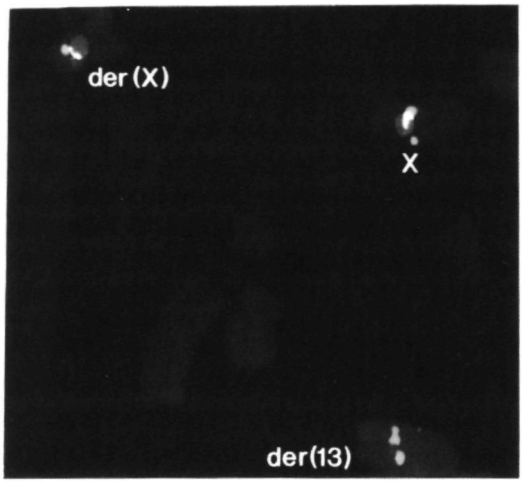


Figure 1. Fluorescence *in situ* hybridization of YAC yXWD940 to metaphase chromosomes of patient PMI. The hybridization signals derived from the YAC clone (green) are located on the normal X chromosome, the derivative X-chromosome and the derivative chromosome 13 indicating that it encompasses the breakpoint. The X centromere is marked in red.

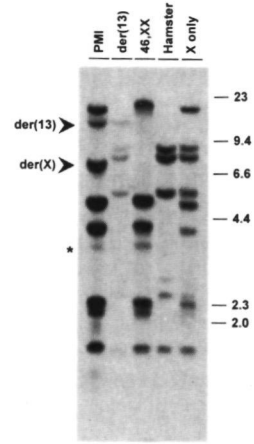


Figure 2. Hybridization of a full length clone of the *DXS6673E* gene to a Southern blot containing *Pvu*II digested DNA from the patient (PMI), a human–hamster hybrid cell line with the derivative chromosome 13 [der(13)], a female control (46,XX), a hamster control and a human–hamster hybrid cell line with the human X-chromosome as its only human chromosomal component (X only). Novel aberrant restriction fragments are seen in DNA from the patient (arrows). The faint cross-hybridizing restriction fragment is labelled with an asterisk.

regionally map the X chromosomal breakpoint by Southern blot analysis and PCR amplification of markers from the Xq13–q21 region. The breakpoint could be assigned to a small segment of Xq13.1 between the gene encoding the gap junction protein connexin 32 (*GJB1*) (18,19) and the cell cycle gene *CCG1* (20) (data not shown). Four YAC clones, two of which were identified with *DXS348* (21,22) and two with *CCG1*, were hybridized *in situ* to metaphase chromosomes of the patient. One of the YACs identified with *CCG1*, clone yWXD940, spans the breakpoint on the X chromosome (Fig. 1). Apart from the two X-centromeric hybridization signals, three signals were seen representing the hybridization of the YAC to the normal X chromosome, the derivative X-chromosome and the derivative chromosome 13 [der(13)], respectively.

Isolation of a cDNA clone that is disrupted by the translocation

By subcloning YAC yWXD940, a partial cosmid contig was generated. Employing Southern blot analysis a cosmid, L2, could be identified that crossed the breakpoint. This cosmid is located approximately 150 kb proximal to the cell cycle gene *CCG1* as estimated from pulsed field gel electrophoresis (data not shown).

The human insert of cosmid L2 was hybridized to a fetal brain cDNA library which resulted in the isolation of 17 cDNA clones. Further screening of the same library with these cDNAs led to the isolation of over 45 homologous, partially overlapping cDNA clones. Hybridization of the clones 50:11 and 50:17 representing the full length cDNA (Fig. 3) to a Southern blot with *Pvu*II digested DNA from the patient, the hybrid cell line carrying the der(13), as well as several controls, clearly showed that a gene represented by these cDNA clones is disrupted by the translocation (Fig. 2). In the DNA of the patient, two aberrant restriction fragments were seen. One of these fragments was also seen in the DNA from the human–hamster hybrid cell line containing the der(13) chromosome. Aberrant bands were also obtained by hybridization of the same probe to a Southern blot containing DNA from the patient and a female control digested with several other enzymes (data not shown). The *DXS6673E* gene, from which these cDNAs are derived, hybridizes to three adjacent *Eco*RI fragments of 6, 10 and 6 kb. The most distal 6 kb *Eco*RI fragment is disrupted by the translocation in the patient (Fig. 3).

Several partially overlapping cDNA clones were characterized by restriction mapping and sequence analysis (Figs 3 and 4) and two of the clones displayed a poly(A) tail at their 3′ end. Also, subclones of cosmid L2 were used to elucidate the genomic structure of the gene. Two cDNA variants of the *DXS6673E* gene were identified. Both variants consist of 25 exons and differ only in their first exon encoding the 5′ untranslated region (exon 1A and 1B, see below and Figs 3 and 4). All splice donor and splice acceptor sites fit the consensus as calculated according to Shapiro and Senapathy (Table 1) (23). The length of the consensus cDNA sequence is 6126 bp (variant 1A) and 5538 bp (variant 1B), respectively. Both splice variants encode a mRNA with an open reading frame of 4074 bp, corresponding to a protein sequence of 1358 amino acids (aa). The complete cDNA sequence of variant 1A has been deposited in GenBank, accession number X95808. A polyadenylation site is located at position 6075 followed by a poly(A) tail 12 bp downstream. In the 5′ untranslated region of the gene, a highly polymorphic GA repeat sequence was identified. PCR analysis revealed that the translocation disrupts exon 1A, hence, the breakpoint is located in the 5′ untranslated region (Fig. 3). The telomere to centromere orientation of transcription could be inferred from hybridization of the 5′ and the 3′ end of the *DXS6673E* gene to DNA from the human–hamster hybrid cell line containing the der(13) chromosome (data not shown).

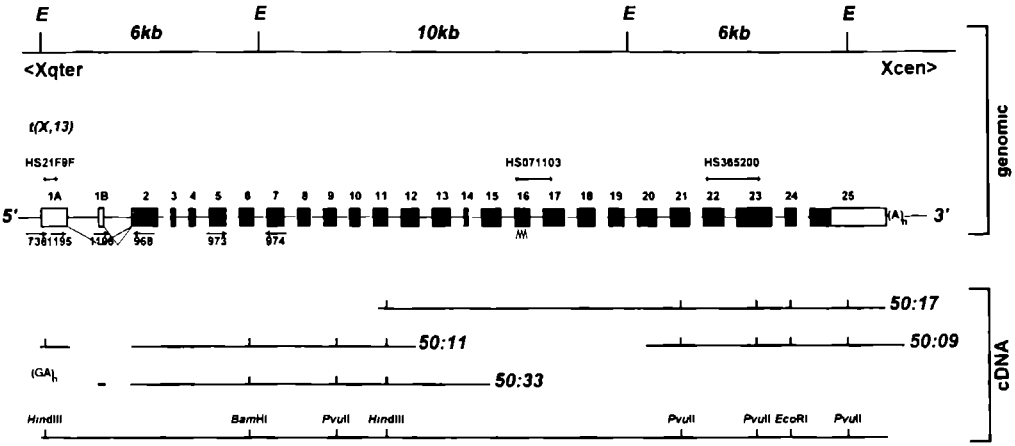
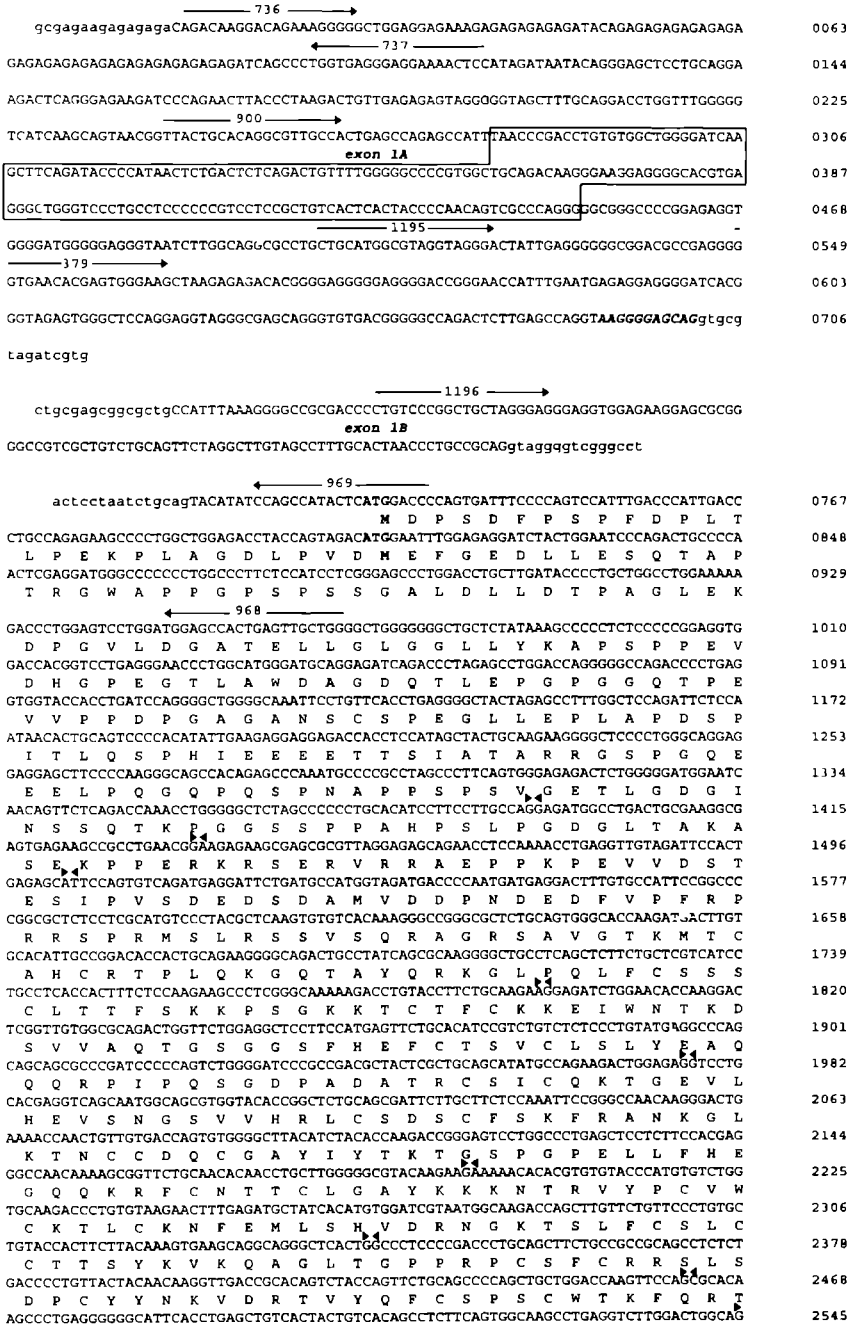


Figure 3 Genomic and cDNA structure of the *DXS6673E* gene spanning the X chromosomal breakpoint. The *DXS6673F* gene is located in three adjacent *Eco*RI fragments of 6, 10 and 6 kb. It consists of 25 exons of which exon 1A and 1B encode two alternative splice variants. The open reading frame is indicated with black boxes. Primers used for RT–PCR analyses are indicated with small arrows beneath the exons. The breakpoint is disrupting exon 1A as indicated with a dotted line. Four cDNA clones (50.09, 50.11, 50.17 and 50.33) representing the entire gene are indicated with bold lines at the bottom of the figure. The position of a highly polymorphic $(GA)_6CATA(GA)_{20}$ repeat in exon 1A is indicated with a dotted line. The identity between three ESTs and the *DXS6673E* gene, as well as the CpG island is indicated with double arrowheads while the putative transmembrane domain is shown with a wavy line. The position of *Eco*RI, *Bam*HI, *Hin*dIII and *Pvu*II restriction sites in the cDNA are indicated on a bar at the bottom of the figure.

Table 1 Splice sites of the *DXS6673E* gene

Exon	Splice donor site[a]	Consensus[b]	Splice acceptor site[a]	Consensus[b]	Position[c]	Exon size
1A RT–PCR	—		CTTGAGCCAG gtaagg	65%	693	
1A cDNA 50.11	—		AGGGGAGCAG gtgcgt	85%	706	
1B	—		CTGGCCGCAG gtgggg	81%		
2	ctcctaatctgcag TACATATCCA	86%	TCCTTGCCAG gtaggt	89%	1392	686 bp
3	tgcttccttgcag GAGATGGCCT	97%	GCCTGAACGG gtgagg	84%	1436	44 bp
4	tttccgaggtacag AAGAGAAGCG	81%	ACTGAGAGCA gtaagt	79%	1503	67 bp
5	ccccacttttctcag TTCCAGTGTC	83%	TCTGCAAGAA gtgcgt	73%	1798	295 bp
6	gttcccacctctag GGAGATCTGG	84%	GACTGGAGAG gtgagg	92%	1976	178 bp
7	tatgtgtgtggcag GTCCTGCACG	82%	GTACAAGAAG gtgggg	81%	2195	219 bp
8	ccccatccteacag AAAAACACAC	87%	GGGCTCACTG gtaggt	81%	2346	151 bp
9	tctctatgcccccag GCCCTCCCCG	93%	CAAGTTCCAG gtactc	68%	2462	116 bp
10	ttccttcccctccag CGCACAAGCC	90%	GGACTGGCAG gtaaga	94%	2549	87 bp
11	tgtcccaacctcag GACCAAGTGT	92%	TGCAGCGAAG gtaagg	95%	2700	151 bp
12	tctctctttccag GCTGTGTGCT	96%	GTACTGCAAG gtaagg	95%	2873	173 bp
13	tttgacatctctcag CTGCCCGGTG	83%	CTCCTGAACA gtgagt	76%	3039	166 bp
14	gttttgatctctag GTCAGTCTCC	94%	CAGCAACACA gtgagg	72%	3101	62 bp
15	ctctgtctttgcag ATCCCTGTGA	92%	AGTCAAACAG gtgagc	91%	3261	160 bp
16	tttcttatccacag AAGAGTGGAA	93%	GCCTATCCCG gtgagt	89%	3374	113 bp
17	attcgtgcctccag GTGCCTGTGC	89%	GACCTTTGTG gtatgc	75%	3549	175 bp
18	tctctgcttcccag ATCTTGTGAG	92%	TTCCCTAAGA gtgagt	97%	3696	147 bp
19	ttcccctttcattag ATCCTCTGGA	79%	CATGAGGAAG gtaggg	85%	3800	104 bp
20	ctcttccccttccag GGTCAAAAGC	95%	CGCTTTGGCC gtaagt	78%	3969	169 bp
21	acatatcactgcag CCAAACCCAT	78%	CATCCAGCAG gtacct	74%	4121	152 bp
22	ttttcttttcccag TACTTGCTGG	91%	CTCCCCAACA gtaagg	75%	4236	125 bp
23	cttgaccattgtag ATACGGTGTT	76%	AAAGGGCGAG gtacga	83%	4491	255 bp
24	tccctcccctatag ACACGGGTCC	81%	TCTCAAAATG gtgagt	89%	4609	128 bp
25	cccgatgttgcag TCCTGAAAGC	78%			6092	1483 bp

[a]Lower case letters indicate intron sequences, upper case are exon sequences.
[b]The percentage similarity to splice consensus sequences was calculated according to Shapiro and Senapathy (23).
[c]Position as calculated from variant 1A (accession number X95808).


736
737
900
exon 1A
1195
379
1196
exon 1B
969
968

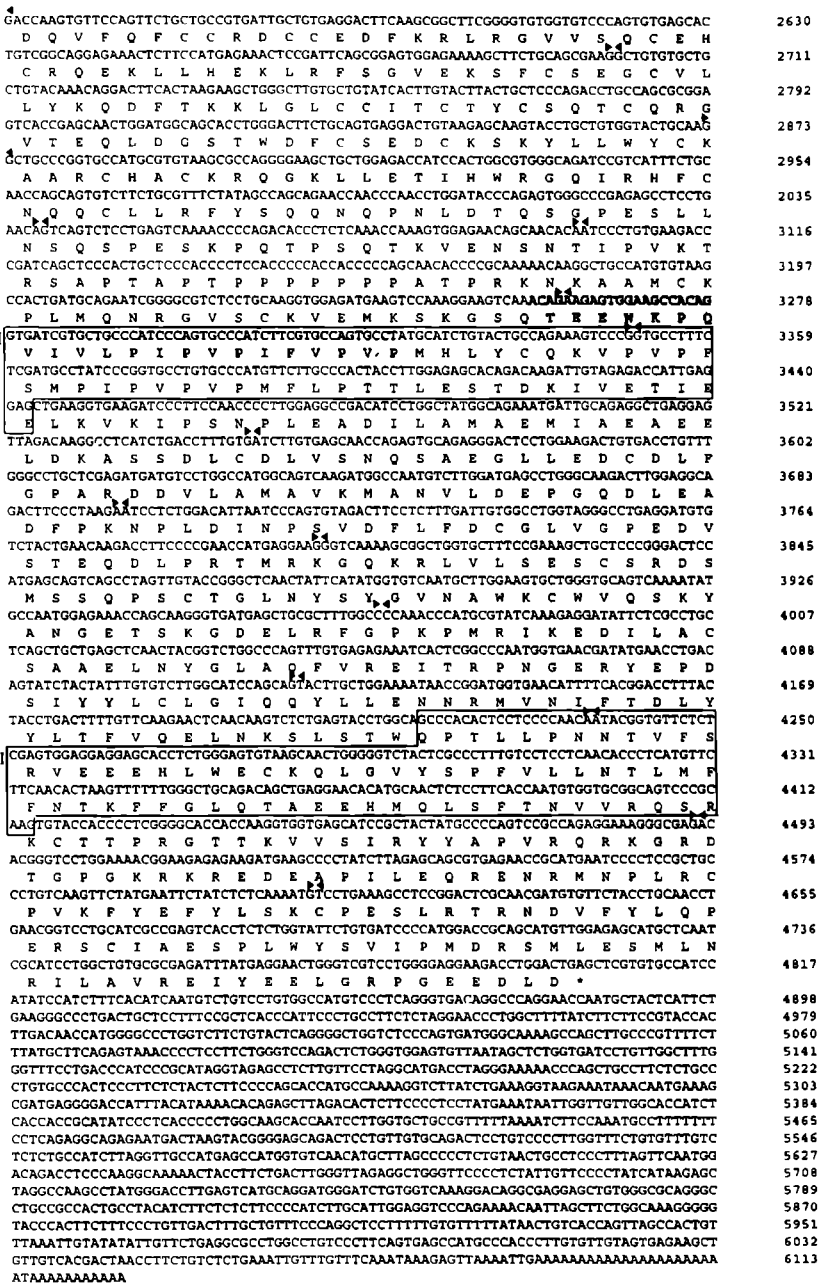


Figure 4 Genomic and cDNA sequences of the *DXS6673E* gene. Genomic sequence of exons 1A, 1B and part of exon 2, cDNA sequence of exons 2–25. Exon sequences are in upper case, intron sequences in lower case. The predicted protein sequence is given in the one letter code. The putative AUG start codons are in bold. The 13 bp difference in exon 1A between RT–PCR products and cDNA clone 50.11 is in bold and italics. Primers used for RT–PCR amplification and for amplification of the GA repetitive sequence in exon 1A are indicated with arrows and their respective numbers. Splice sites are indicated with double arrowheads while the regions identical to the ESTs HS21F9F (I), HS071103 (II) and HS365200 (III) are boxed. The putative transmembrane domain is shaded.

Exon 1A was only found in a single cDNA clone, 50:11. To confirm that this putative exon is transcribed, RT–PCR on total RNA from an EBV-transformed human cell line was performed (Figs 3 and 4). The PCR product generated with primers from exon 1A and 2 was sequenced and found to match the sequence of the cDNA clone except for a deletion of 13 nucleotides proximal to the splice donor site of exon 1A. This 13 bp discrepancy may be the result of alternative splicing. RT–PCR products generated with the same primers from total RNA of adult muscle, adult kidney and fetal brain gave identical results. Also, the remaining sequence of exon 1A could be amplified by RT–PCR thereby confirming that this is a true exon (data not shown). RT–PCR with primers from exon 1B and exon 2 yielded a DNA fragment of the expected length while RT–PCR analysis with primers from exon 1A and exon 1B did not yield a PCR product. This indicates that exon 1B is part of this gene and that exons 1A and 1B represent alternative starts of transcription (Figs 3 and 4).

A Fasta sequence homology search showed an almost complete identity of the 3′ end of the *DXS6673E* gene with two ESTs (HS04328 and HS448109) and of the 5′ end of the *DXS6673E* gene with a third EST; HS21F9F. Also, weaker identity was found with two other ESTs at two different positions in the ORF of the *DXS6673E* gene (HS071103 and HS365200) (Figs 3 and 4). A BestFit-GCG package comparison (24) of the corresponding polypeptide sequences with the *DXS6673E* protein revealed 58% identity over 56 aa for HS071103 and 58% identity over 66 aa for HS365200, respectively (Figs 3 and 4). No significant homologies were identified when comparing the *DXS6673E* protein sequence to sequences from the protein database (Swiss-Prot). Sequence analysis done according to Kyte and Doolittle (25) suggested that the protein is largely hydrophilic with one putative transmembrane domain between aa positions 845 and 865 (Figs 3 and 4).

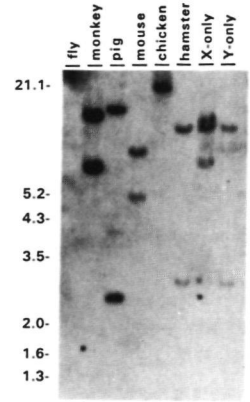


Figure 5. Hybridization of clone 50:17 to a Zoo blot containing *Eco*RI digested DNA from several organisms. In all animals tested except fish (not shown) and fly hybridization signals remain visible, even with stringent hybridization at 65°C and washing conditions (0.1× SSC, 0.1% SDS at 65°C).

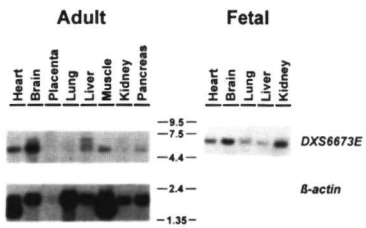


Figure 6. Hybridization of clone 50:17 to an adult and fetal Northern blot containing poly (A)+ RNA from several tissues. In all tissues except placenta multiple hybridization signals are visible. The gene is highly expressed in brain and muscle and the expression level of the different products varies with the tissue in which it is expressed. In contrast to the adult tissues, only one product is seen in fetal tissues. As a control a β-actin cDNA clone was hybridized to the adult Northern blot.

Conservation and expression of the *DXS6673E* gene

cDNA clone 50:17 was hybridized to a 'Zoo blot' containing *Eco*RI digested DNA from various animal species (Fig. 5). Even at stringent conditions of 0.1× SSC and 0.1% SDS at 65°C, hybridization signals were seen in all animals except fly and fish (not shown). The gene could be mapped to the human X chromosome by analyzing a human–hamster somatic cell hybrid with a single human X chromosome as its only human chromosomal component.

Hybridization of the same cDNA clone to Northern blots containing poly(A)+ RNA from different fetal and adult tissues revealed a transcript of approximately 6.0 kb in almost all tissues tested (Fig. 6) which is in keeping with the size of the cDNA consensus sequence. The expression was found to be most abundant in brain, moderate in muscle and heart and low in several other tissues. In contrast to the Northern blot with RNA from fetal tissues, at least three different transcripts of 6, 6.5 and 7 kb were seen on the Northern blot containing RNA from adult tissues. The expression level of these different transcripts varied between the tissues tested.

X-inactivation of the *DXS6673E* gene and gene expression in the patient

To determine whether this gene is subject to X-inactivation, its expression was studied in human–hamster somatic cell hybrids carrying an active or inactive human X chromosome, respectively. RT–PCR experiments were performed with primers derived from exon 5 and 7; as a control, we used primers specific for the *SMCX* gene that is not subject to X-inactivation (26). In the human–hamster somatic cell hybrid with an active human X chromosome, PCR products were found for both genes, but in the cell line with the inactive X chromosome only a product was found when using the *SMCX*-specific primers (Fig. 7). These results indicate that the *DXS6673E* gene underlies X-inactivation.

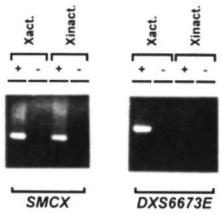


Figure 7. RT–PCR analysis with (+) without (–) MMLV reverse transcriptase of the *DXS6673E* gene on a human–hamster somatic cell hybrid containing an active (Xact) and an inactive (Xinact) human X chromosome. The gene is subject to X inactivation. As a control, primers from the human *SMCX* gene were used (26).

Previously, replication studies had been performed in 100 peripheral blood lymphocytes (PBL) of the patient. Consistently, the normal X chromosome was found to be late replicating indicating preferential X-inactivation of the normal X chromosome (15). It was expected, therefore, that the *DXS6673E* gene would not be expressed in somatic cells of the patient. (Nested) RT–PCR experiments on RNA isolated from peripheral blood and EBV transformed lymphoblastoid cell lines of the patient and a female control were performed with primers from exon 1A, exon 1B and exon 2. RT–PCR experiments with primers from exon 1B and exon 2 as well as primers from exon 1A and exon 2 that are located proximally to the breakpoint show normal gene products (Fig. 8A). In contrast, a nested RT–PCR with primers from exon 1A and exon 2 that span the breakpoint, failed to result in a normal gene product on RNA of the patient (Fig. 8A).

DISCUSSION

The positional cloning of numerous disease genes has been facilitated by the presence of chromosome aberrations that are associated with specific disorders (12). The incidence of balanced chromosome rearrangements among newborns is approximately 1:3000 and 6% of these rearrangements are associated with abnormal phenotypes (13). In 50% of these cases, the aberrant clinical phenotype may be causally related to the karyotypic changes. This proportion may even be higher for X-autosome translocations because in males truncation of X-chromosomal genes will result in a functional nullisomy. Usually, the same is true for females with balanced X-autosome translocations because of preferential inactivation of the normal X (13,14,27). The molecular characterization of balanced X chromosomal

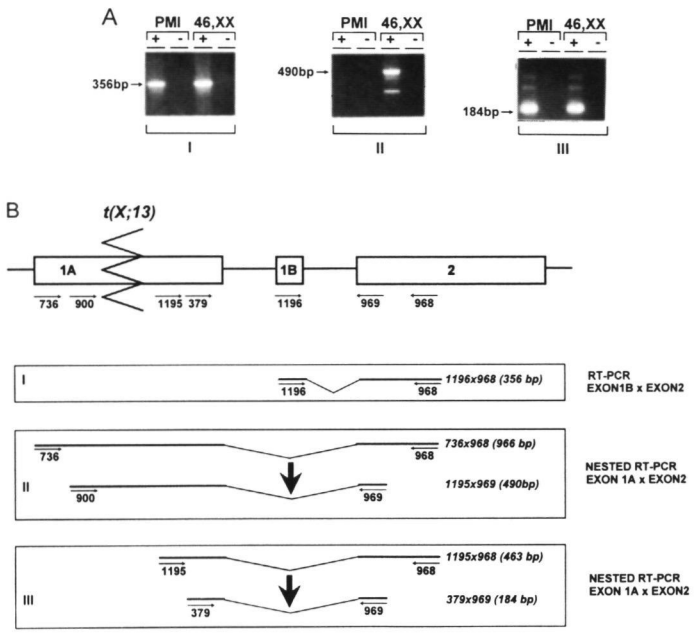


Figure 8. RT–PCR analysis of the *DXS6673E* gene in patient PMI. (**A**) (Nested) RT–PCR analysis with (+) and without (–) MMLV reverse transcriptase of the *DXS6673E* gene on total RNA from the patient (PMI) and a female control (46,XX) with the primer combinations 1196×968 (I) to amplify variant 1B. To amplify variant 1A the primers 900×969 (II) and 379×969 (III) were used. (**B**) Schematic overview of exons 1A, 1B and 2 of the *DXS6673E* gene with the primers used for RT–PCR analysis. The exons are boxed and shaded, the breakpoint is represented by a wavy line and the primers are indicated with small arrows. Nested RT–PCR products of different primer combinations are indicated with solid lines beneath the exons. At their right sides, the primer combination and the expected length of the product is indicated. Large arrows indicate the primer combinations used for nested PCR experiments on primary RT–PCR products. Each distinctive combination is boxed: product I is derived from exon 1B and exon 2, product II is a nested PCR product of exon 1A and exon 2 that spans the translocation, while product III represents a nested PCR product generated with primers from exon 1A and exon 2 that do not span the breakpoint.

rearrangements associated with MR therefore is a promising strategy for the identification of new candidate genes for XLMR

Here we have identified and characterized an X chromosomal gene *DXS6673E* that is disrupted in its 5′ non coding region by an X 13 translocation in a mentally retarded female The gene encodes two mRNAs of 6126 bp and 5538 bp respectively and consists of 25 exons spread over approximately 22 kb of genomic DNA It contains two alternative first exons both encoding a 5 untranslated region which may be under the control of different tissue specific promotors Analysis of cDNA clones and RT–PCR experiments demonstrated that these sequences are part of the gene Moreover the splice junctions of both exons fit the consensus sequence There is a 13 bp discrepancy between the cDNA clone and the RT–PCR product of the first exon from various tissues and cell lines at the splice donor site As both variants show convincing similarity with the consensus splice donor site again this may be due to alternative splicing For these variants an open reading frame of 1358 aa was identified that starts in exon 2 and continues through all downstream exons There are two putative AUG start codons at position 726 and 807 respectively (Fig 4) The second AUG fits the Kozak consensus sequence better than the first which lacks a purine residue at position −3 (28) Therefore we believe that the second AUG is the translation initiation site Identity was found with five ESTs when comparing the *DXS6673E* gene to the database three of which (HS04328 HS448109 and HS21F9F) encode the same gene Interestingly HS21F9F was isolated by using a methylated DNA binding column that selects for CpG like sequences (29) Moreover this region of identity colocalizes with the breakpoint region However a CpG plot (GCG package) did not reveal the presence of a CpG island in exon 1A (24 30) In contrast a 300 bp sequence starting in intron 1A and extending through exon 1B into intron 1B (Fig 3) was identified that meets all criteria for a CpG island (data not shown) The two other ESTs may be derived from homologous domains of related genes One of these ESTs HS071103 is similar to the putative transmembrane domain of the *DXS6673E* protein which exhibits highly conserved stretches of neutral and hydrophobic residues with a proline residue on every second position The two ESTs are derived from different tissues HS365200 from fetal liver and spleen and HS0711023 from postnatal whole brain respectively Still they may represent different parts of one gene

Northern blots containing poly(A)$^+$ RNA from fetal tissues only show one hybridization signal which corresponds to the length of the cDNA As most of the clones from the fetal brain cDNA library contained exon 1B we assume that this signal represents splice variant 1B and that variant 1A is expressed at a low level during fetal development Moreover the apparent lack of a CpG island associated with exon 1A and the presence of an exon 1B associated CpG island supports the ubiquitous expression of variant 1B and a tissue specific expression of variant 1A as all housekeeping genes and only 40% of all tissue restricted genes exhibit 5′ CpG islands (30–32) In contrast to fetal RNA poly(A)$^+$ RNA from adult tissues yielded multiple hybridization signals which may represent different splice variants or homologous genes The presence of different cDNA variants the identity with two ESTs and a faint autosomal restriction fragment seen on the Southern blot (Fig 2) can be reconciled with both possibilities Although the expression of the gene seems to be ubiquitous the highest expression is found in brain and striated muscles Hybridization of the *DXS6673E* gene to DNA from various animal species indicates that the gene is highly conserved among vertebrates and suggests that it plays an important part

In the great majority of females with balanced X autosome translocations the non rearranged X chromosome is preferentially inactivated (14 33) The same holds true for this patient as judged from replication studies on PBLs Our RT–PCR experiments have shown that no products were generated when *DXS6673E* specific primers were used from both sides of the breakpoint which is consistent with a complete inactivation of the non rearranged *DXS6673F* allele However when using primer combinations located proximally from the breakpoint normal gene products were identified suggesting that variant 1A of the *DXS6673E* gene is under the control of chromosome 13 sequences This may also be true for variant 1B as exon 1B is located approximately 600 bp downstream from the breakpoint Therefore the phenotype of the patient may be due to aberrant temporal and spatial expression or silencing of the *DXS6673E* gene Alternatively the translocation may directly disrupt a gene on chromosome 13 may place a chromosome 13 gene under the control of X chromosomal sequences or indirectly interfere with proper expression of other genes on chromosome 13 as a result of spreading of X inactivation into the autosomal segment of the der(13) If a gene on chromosome 13 is disrupted by the breakpoint the phenotype of the patient could be the result of haploinsufficiency (27) or if the other allele is mutated the translocation may unmask its heterozygosity (34) Large deletions resulting in partial chromosome 13 monosomies have been described in a number of patients with 13q syndrome (reviewed in refs 35 and 36) and association of these deletions with disease phenotypes may support the presence of genes in this region that are sensitive to haploinsufficiency This syndrome includes MR as well as various malformations and the major manifestations in this syndrome seem to be associated with a deletion of the 13q32 band

The breakpoint on chromosome 13 has been mapped between the loci D13S755 and D13S747 by PCR analysis of the der(13) cell line (data not shown) This segment is located up to 3 centimorgans (cM) proximal to the endothelin receptor B (*EDNRB*) gene which was found to be mutated in patients with Hirschsprung disease (HSCR2) (37 38) Although hypopigmentation is also one of the clinical features of HSCR2 an effect of the translocation on the expression of the *EDNRB* gene expression is not very likely Likewise inactivation of the *EDNRB* locus cannot be ascribed to spreading of X inactivation into the chromosome 13 since the X inactivation centre (39) is located on the der(13) and the *EDNRB* gene on the der(X) The observed cutaneous spotting may indicate that there is a small proportion of cells in which the der(X) is inactivated instead of the normal X (40) Although we cannot exclude the possibility that the MR in this patient is due to haploinsufficiency resulting from disruption of a gene on chromosome 13 the disruption of the *DXS6673E* locus remains by far the most plausible cause for MR in this patient A recent overview by Neri *et al* (10) lists eight families with non syndromic MR where the mapping interval includes the Xq13 band (MRX1, 4, 5 7 8 12 13 and 17) Also multiple syndromic types of XLMR have been mapped to the pericentromeric region of the X chromosome Mutation screening in these families should clarify the role of the *DXS6673E* gene in the aetiology of XLMR

MATERIALS AND METHODS

Patient PMI; t(X;13)(q13,q31)

Patient PMI is a mentally retarded girl carrying a *de novo* balanced X 13 translocation She was the second of healthy non consanguineous parents At the age of 1 she suffered from hyperthermic convulsions Apart from mental retardation (IQ<45 WISC R scale) she suffers from scoliosis and spotty hypopigmentation of the skin She also presents with slight facial asymmetry and clinodactily (MRX39) (15 17)

Fluorescence *in situ* hybridization (FISH)

FISH procedures used were essentially as described elsewhere (41–43) The X chromosome centromere specific alphoid sequence probe pBAMX5 was labelled with Fluorolink Cy3 dCTP (BDS Inc Pittsburg) while the YAC clones were labelled with biotin 14 dATP using a nick-translation kit (Gibco Life Technologies) 200 ng labelled YAC probe was dissolved in 6 µl of a hybridization solution containing 50% v/v deionized formamide 10% w/v dextran sulphate 2× SSC and 1% v/v Tween 20 at pH 7 0 as well as 10 mg Cot 1 DNA (Gibco Life Technologies) Prior to hybridization the probe was denatured at 80°C for 10 min chilled on ice and incubated for 30 min at 37°C allowing preannealing For the pBAMX5 probe 20 ng of DNA was used in 6 µl of the hybridization solution without competitor DNA Metaphase spreads were prepared using standard procedures After denaturation of the slides probe incubations were carried out under an 18×18 mm coverslip in a moist chamber for 45 h

Immunocytochemical detection of the hybridizing probes was achieved as described elsewhere (41–43)

YAC clone and construction of a cosmid contig

YAC yWXD940 is a 350 kb YAC containing the *CCG1* gene (20) Yeast cell culturing and DNA isolation were done as described elsewhere (44) A partial cosmid contig from YAC yWXD940 was generated essentially as described elsewhere (45)

Southern blot analysis

Methods employed for the isolation of high molecular weight DNA restriction endonuclease digestion as well as separation and blotting of DNA fragments have been described elsewhere (45) Hybridization of the cosmid inserts in the presence of excess human competitor DNA was done essentially as described by Blonden *et al* (46) Hybridized filters were washed at 65°C with 40 mM Na_2HPO_4 (pH 7 2)/0 5% (w/v) SDS for 3×5 min and 1×30 min Autoradiography took 4–16 h at –70°C using two intensifying screens

Northern blot analysis

Fetal and adult multiple tissue northern blots (Clontech) were hybridized in a solution containing 5× SSPE 10× Denhardt s solution 100 mg/ml herring sperm DNA 50% formamide and 2% SDS for 18 h at 42°C after prehybridization for 3 h under the same condition Hybridized filters were washed 30 min in 2× SSC 0 05% SDS at room temperature and 30 min at 50°C in 0 1× SSC 0 1% SDS Autoradiography took 16–40 h at –70°C using two intensifying screens

cDNA screening and sequence analysis

A λZapII human fetal brain cDNA library (Stratagene) was screened by standard methods (47) and Bluescript KS(+) plasmids containing cDNA inserts were isolated by *in vivo* excision according to the manufacturer s instructions

Double stranded sequence analysis was performed on DNA isolated from the cDNA clones and on PCR amplified DNA products using T3 and T7 oligonucleotides as well as gene specific oligonucleotides (Isogen Biosciences the Netherlands) Plasmid DNA was isolated using a Qiaprep 8 kit (Qiagen) while PCR products were first separated on an agarose gel and isolated with a gel extraction kit (Qiagen) Sequence analysis was performed radioactively with the T7 sequencing Kit (Pharmacia Biotech) and in addition with the *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) on an Applied Biosystems 373A DNA sequencer according to the manufacturer s instructions

RT–PCR analysis

Total RNA was isolated from EBV transformed cell lines fetal brain adult muscle and liver with RNAzol according to the manufacturer s instructions (Cinna Biotecx) For synthesis of cDNAs 200 ng of total RNA was reversed transcribed with MMLV reverse transcriptase essentially as described elsewhere (48) The cDNA products were directly used as template for PCR amplification in a buffer containing 10 mM Tris 50 mM KCl 0 01% gelatine 2 mM $MgCl_2$ (*DXS6673E*) or 3 mM $MgCl_2$ (*SMCX*) 10 mM dNTPs 250 ng of each oligonucleotide 2 5 *Taq* DNA polymerase (Perkin Elmer) Amplification was done in 30 (*DXS6673E*) or 35 (*SMCX*) cycles of 1 min 94°C 1 min 60°C and 1 5 min 72°C Inactivation studies of the *DXS6673E* gene were done with primers 973 (5′ TGCTCGTCATCCTGCCTCAC 3′) and 974 (5′ ACACTGGTCACAACAGTTGG 3′) amplifying 380 bp from exons 5 and 6 For expression studies of exon 1A the following primer combinations located on both sides of the breakpoint were used primers 736 (5′ AGACAAGGACAG AAAGGGGG 3′) and 968 (5′-CCAGCAACTCAGTGGC TCCA 3′) followed by a nested PCR with primers 900 (5 TTACTGCACAGGCGTTGCCA 3′) and 969 (5′ GGGTCCA TGAG TATGGCTGG 3′) amplifying 490 bp of exon 1A and exon 2 Additionally exon 1A sequences located proximally from the breakpoint were amplified with the primers 1195 (5 CTGCATGGCGTAGGTAGGGA-3′) and 968 followed by a nested amplification with the primers 379 (5′ GGTGAACACGA GTGGGAAGC 3′) and 969 (184 bp) Expression of exon 1B was studied with the primers 1196 (5 CTGTCCCGGCTGCTAGG GAG 3′) and 968 amplifying a product of 356 bp Amplification of *SMCX* cDNA was done with the oligonucleotides S40HX (5′ GAAGGTGGAGTCAACATCGCCT 3′) and S41HX (5 AG CCATCACACAGCAGGAGC 3′) (26)

ACKNOWLEDGEMENTS

The authors are indebted to Mrs S D van der Velde Visser and E Boender van Rossum for expert technical assistance We thank D Rohme Mrs Y J M de Kok and A Kataki for their contributions to this study and J Leunisse for his contribution to homology searches The research of FPM C has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences This work was supported by the Netherlands

Organization of Scientific Research (NWO grant 960–10–809) and the Dutch Preventiefonds (grant 28 2447)

ABBREVIATIONS

aa amino acid cM centimorgan *EDNRB* endothelin receptor B gene HSCR2 Hirschsprung disease 2, MR, mental retardation XLMR X-linked mental retardation YAC yeast artificial chromosome

REFERENCES

1 Herbst D S and Miller J R (1980) Nonspecific X linked mental retardation II the frequency in British Columbia *Am J Med Genet* **7**, 461–469
2 Opitz J M (1986) Editorial comment on the gates of hell and a most unusual gene *Am J Med Genet* **23**, 1–10
3 Opitz J M (1987) Erratum *Am J Med Genet* **26**, 37
4 Kerr B Turner G Mulley J Gedeon A and Partington M (1991) Non specific X linked mental retardation *J Med Genet* **28**, 378–382
5 Glass I A (1991) X linked mental retardation *J Med Genet* **28**, 361–371
6 Verkerk A J M H Pieretti M Sutcliffe J S Fu Y Kuhl D P A Pizzuti A Reiner O Richards S Victoria M F Zhang F Eussen B E van Ommen G B Blonden L A J Riggins G J Chastain J L Kunst C B Galjaard H Caskey C T Nelson D L Oostra B A and Warren S T (1991) Identification of a gene (*FMR 1*) containing a CGG repeat coincident with a fragile X breakpoint cluster region exhibiting length variation in fragile X syndrome *Cell* **65**, 905 914
7 Jouet M Rosenthal A Armstrong G MacFarlane J Stevenson R Paterson J Metzenberg A Ionasescu V Temple K and Kenwrick S (1994) X linked spastic paraplegia (SPG1) MASA syndrome and X linked hydrocephalus result from mutations in the *L1* gene *Nature Genet* **7**, 402–407
8 Vits L van Camp G Coucke P Fransen E De Boulle K Reyniers E Korn B Poustka A Wilson G Schrander Stumpel C Winter R M Schwartz C and Willems P J (1994) MASA syndrome is due to mutations in the neural cell adhesion gene *L1CAM Nature Genet* **7**, 408–413
9 Gibbons R J Picketts D J Villard L and Higgs D R (1995) Mutations in a putative global transcriptional regulator cause X linked mental retardation with α thalassemia (ATR X syndrome) *Cell* **80**, 837–845
10 Neri G Chiurazzi P Arena J F and Lubs H A (1994) XLMR genes update 1994 *Am J Med Genet* **51**, 542–549
11 Ropers H van der Maarel S Knoers N Kremer H Smits A Hamel B Cremers F P M Gilgenkrantz S Philippe C Monaco T Ishikawa Brush Y Smeets D and Mariman E (1994) Unspecific X linked mental retardation clinical genetic and molecular studies *Am J Hum Genet* **55**, A49
12 Collins F S (1995) Positional cloning moves from perditional to traditional *Nature Genet* **9**, 347–350
13 Warburton D (1991) De novo balanced chromosome rearrangements and extra marker chromosomes identified at prenatal diagnosis clinical significance and distribution of breakpoints *Am J Hum Genet* **49**, 995 1013
14 Schmidt M and Du Sart D (1992) Functional disomies of the X chromosome influence the cell selection and hence the X inactivation pattern in females with balanced X autosome translocations a review of 122 cases *Am J Med Genet* **42** 161 169
15 Teboul M Mujica P Chery M Leotard B and Gilgenkrantz S (1989) Translocations X autosomes equilibrees et retard mental Contribution a la cartographie des retards mentaux lies a L X (à l exclusion de L X FRA) *J Genet Hum* **37**, 179 195
16 Abelovich D Dagan J Kimchi Sarfaty C and Zlotogora J (1995) Paracentric inversion X(q21 2q24) associated with mental retardation in males and normal ovarian function in females *Am J Med Genet* **55**, 359 362
17 Philippe C Cremers F P M Chery M Bach I Abbadi N Ropers H and Gilgenkrantz S (1993) Physical mapping of DNA markers in the q13–q22 region of the human X chromosome *Genomics* **17**, 147–152
18 Cotton J A Lafreniere R G Begy C R Loch Caruso R Willard H F and Glover T W (1992) Refined localization of human connexin32 gene locus GJB1 to Xq13 1 *Genomics* **13**, 479–480
19 Raimondi E Grudi S Moralli D De Carli L Malcovati M Simonic T and Tenchini M L (1992) Assignment of the human connexin 32 gene (GJB1) to band Xq13 *Cytogenet Cell Genet* **60**, 210–211
20 Sekiguchi T Miyata T and Nishimoto T (1988) Molecular cloning of the cDNA of human X chromosomal gene (CCG1) which complements the temperature sensitive G_1 mutants tsBN462 and ts13 of the BHK cell line *EMBO J* **7**, 1683–1687
21 Barker D F Dietz Band J N Donaldson C W Andersen W L Turco A F Pole A R Willard H F and Vincent A (1989) Further isolation characterization and physical localization of X chromosome RFLP markers comparing VNTR directed and random isolation strategies *Cytogenet Cell Genet* **51** 958
22 Lafreniere R G Brown C J Powers V E Carrel L Davies K E Barker D F and Willard H F (1991) Physical mapping of 60 DNA markers in the p21 1→q21 3 region of the human X chromosome *Genomics* **11**, 352–363
23 Shapiro M B and Senapathy P (1987) RNA splice junctions of different classes of eukaryotes sequence statistics and functional implications in gene expression *Nucleic Acids Res* **15**, 7155 7174
24 Devereux J Haeberli P and Smithies O (1984) A comprehensive set of sequence analysis programs for the VAX *Nucleic Acids Res* **12**, 387–395
25 Kyte J P and Doolittle R F (1982) A simple method for displaying the hydropathic character of a protein *J Mol Biol* **157**, 105 132
26 Agulnik A I Mitchell M J Mattei M G Borsani G Avner P A Lerner J L and Bishop C F (1994) A novel X gene with a widely transcribed Y linked homologue escapes X inactivation in mouse and human *Hum Mol Genet* **3**, 879–884
27 Tommerup N (1993) Mendelian cytogenetics Chromosome rearrangements associated with Mendelian disorders *J Med Genet* **30**, 713 727
28 Kozak M (1987) An analysis of 5 noncoding sequences from 699 vertebrate messenger RNAs *Nucleic Acids Res* **15**, 8125–8148
29 Cross S H Charlton J A Nan X and Bird A P (1994) Purification of CpG islands using a methylated DNA binding column *Nature Genet* **6**, 236–244
30 Gardiner Garden M and Frommer M (1987) CpG islands in vertebrate genomes *J Mol Biol* **196**, 261–282
31 Larsen F Gundersen G Lopez R and Prydz H (1992) CpG islands as gene markers in the human genome *Genomics* **13**, 1095–1107
32 Cross S H and Bird A P (1995) CpG islands and genes *Curr Opin Genet Dev* **5**, 309 314
33 Mattei M G Mattei J F Ayme S and Giraud F (1982) X autosome translocations cytogenetic characteristics and their consequences *Hum Genet* **61**, 295 309
34 Buhler E M (1983) Unmasking of heterozygosity by inherited balanced translocations Implications for prenatal diagnosis and gene mapping *Ann Genet* **26**, 133–137
35 Brown S Gersen S Anyane Yeboa K and Warburton D (1993) Preliminary definition of a critical region of chromosome 13 in q32 report of 14 cases with 13q deletions and review of the literature *Am J Med Genet* **45** 52–59
36 Brown S Russo J Chittavat D and Warburton D (1995) The 13q syndrome the molecular definition of a critical deletion region in band 13q32 *Am J Hum Genet* **57**, 859–866
37 Washington S S Warburton D and Chakravarti A (1995) Report of the second international workshop on human chromosome 13 mapping 1994 *Cytogenet Cell Genet* **70**, 1–22
38 Puffenberger E G Hosoda K Washington S S Nakao K deWit D Yanagisawa M and Chakravarti A (1994) A missense mutation of the endothelin B receptor gene in multigenic Hirschsprung s disease *Cell* **79**, 1257–1266
39 Brown C J Hendrich B D Rupert J L Lafreniere R G Xing Y Lawrence J and Willard H F (1992) The human *XIST* gene analysis of a 17 kb inactive X specific RNA that contains conserved repeats and is highly localized within the nucleus *Cell* **71**, 527–542
40 Thomas I T Frias J L Cantu E S Lafer C Z Flannery D B and Graham J G Jr (1989) Association of pigmentary anomalies with chromosomal and genetic mosaicism and chimerism *Am J Hum Genet* **45**, 193–205
41 Suijkerbuijk R F van de Veen A Y van Echten J Buys C H C M de Jong B Oosterhuis J W Warburton D A Cassiman J J Schonk D and Geurts van Kessel A (1991) Demonstration of the genuine iso 12p character of the standard marker chromosome of testicular germ cell tumors and identification of further chromosome 12 aberrations by competitive in situ hybridization *Am J Hum Genet* **48**, 269 273

42 Sinke R J Suijkerbuijk R F Herbergs J Janssen H Cassiman J J and Geurts van Kessel A (1992) Generation of a panel of somatic cell hybrids containing fragments of human chromosome 12p by X ray irradiation and cell fusion *Genomics* **12**, 206–213
43 Suijkerbuijk R F Looijenga L de Jong B Oosterhuis J W Cassiman J J and Geurts van Kessel A (1992) Verification of isochromosome 12p and identification of other chromosome 12 aberrations in gonadal and extragonadal human germ cell tumors by bicolor double fluorescence in situ hybridization *Cancer Genet Cytogenet* **6**, 8–16
44 Green E D and Olson M V (1990) Systematic screening of yeast artificial-chromosome libraries by use of the polymerase chain reaction *Proc Natl Acad Sci USA* **87**, 1213–1217
45 Huber I Bitner Glindzicz M de Kok Y J M van der Maarel S M Ishikawa Brush Y Monaco A P Robinson D Malcolm S Pembrey M E Brunner H G Cremers F P M and Ropers H (1994) X linked mixed deafness (DFN3) cloning and characterization of the critical region allows the identification of novel microdeletions *Hum Mol Genet* **3**, 1151–1154
46 Blonden L A J den Dunnen J T van Paassen H M B Wapenaar M C Grootscholten P M Ginjaar H B Bakker E Pearson P L and van Ommen G J B (1989) High resolution deletion breakpoint mapping in the DMD gene by whole cosmid hybridization *Nucleic Acids Res* **17**, 5611–5621
47 Maniatis T Fritsch E F Sambrook J (1982) *Molecular Cloning A Laboratory Manual* Cold Spring Harbor NY Cold Spring Harbor Laboratory
48 Kalscheuer V M Mariman E C Schepens M T Rehder H and Ropers H (1993) The insulin like growth factor type 2 receptor gene is imprinted in the mouse but not in humans *Nature Genet* **5**, 74–78

CHAPTER 8

GENERAL DISCUSSION

8. General Discussion

In this study we have set out to identify candidate genes for X chromosomal deafness (DFN3) and mental retardation (XLMR) through the molecular analysis of X chromosomal rearrangements in patients with DFN3 or MR. This analysis not only resulted in the identification of the first deletion in Xq21 associated with MR and CHM only, which underlined the presence of a MR gene in this chromosomal segment, but also enabled us to identify the gene underlying DFN3. This gene, *POU3F4*, encodes a member of a family of POU domain containing proteins which have transcriptional regulating properties.

Moreover, as a first step towards the identification of novel genes for XLMR, we mapped four X chromosomal breakpoints in mentally retarded females with balanced X;a translocations. Detailed analysis of one of these translocations resulted in the identification of an X chromosomal gene that is disrupted by the breakpoint.

8.1 POU Genes: Roles in Inner Ear and Brain Development

A special subclass of transcriptional regulators are the POU-domain containing proteins (reviewed in 1,2). These proteins have DNA and protein binding capacities and may act as monomers, homodimers or heterodimers with other POU proteins. Members of this protein family show different expression patterns during development and in various adult tissues. They may act as positive or negative transcription factors. POU proteins consist of two DNA binding domains, the amino-terminal POU specific (POU_s), and the carboxy-terminal POU homeo (POU_h) domain which are separated by a variable linker. Both domains are required for the specificity and capacity of DNA binding. The homeodomain is likely to consist of three α helices of which the latter two form a helix-turn-helix (HTH) motif which is used for DNA recognition, and its three-dimensional structure is probably similar to that of classical eukaryotic homeodomains. Recently, it was shown that the POU_s domain of one member of the POU protein family, *Oct-1*, consists of four α helices and its structure is highly similar to the λ and 434 bacteriophage repressors (3,4). The POU protein family is subdivided into 6 subclasses based on sequence homologies in the amino and carboxy terminal regions of the POU-domain, and for several of them, expression in the inner ear has been demonstrated (5). One of these POU proteins, *Brn 3-1* (*Pou4f3*), is strongly expressed in the developing rat cochlea, and its expression continues in adult cochlear and vestibular hair cells of the inner ear. This expression patterns suggests a pivotal role for *Brn 3-1* in inner ear function. Indeed, the deletion of the *Brn 3-1* gene in knock-out mice results in complete deafness due to the lack of inner ear hair cells and supporting cells with subsequent loss of cochlear and vestibular ganglia (6). Moreover, these mice show deficits in balance and coordination, and are hyperactive. The human homologue maps to 5q21-q35, and a form of late-onset degenerative

deafness has been mapped to 5q31. Therefore, *POU4F3* is a strong candidate for this form of deafness. Interestingly, deletion of another member of this family, *Brn.3-2*, results in the loss of a large proportion of retinal ganglion cells in *Brn.3-2*$^{-/-}$ mice although its expression is, like *Brn.3-1*, not confined to this tissue (6,7).

BRN4, or *POU3F4*, belongs to the third class (*POU* domain, Class *3*, *F*actor *4*), has no introns, and contains two long A-rich stretches in the 3' noncoding region which indicates that, as all members of this subclass, it has evolved through retrotransposition. The mouse homologue, *Pou3f4*, has been mapped between the *DXMit6* marker and the proteolipid protein locus *Plp*, a region which is evolutionary conserved between mice and humans (8). Moreover, the rat homologue, *RHS2*, is prominently expressed in the otic vesicle between developmental days 13.5 and 19.5 after conception (9). These data strongly suggested a possible role for *POU3F4* in DFN3. Indeed, the presence of deletions of *POU3F4*, as well as four nonsense and five missense mutations in *POU3F4* in patients with DFN3, but not in healthy controls or patients with other forms of X-linked deafness without a temporal bone defect, provided convincing evidence for a causal role of POU3F4 in DFN3 (*chapter 6*, 10,11). It is interesting that all missense mutations are located in the POU_h domain and that no mutations have been identified in other parts of the gene. So far, only one other member of the POU protein family, *POU1F1 (PIT1)*, has been implicated in human disease (12-15). Mutations in *PIT1* give rise to a syndrome of congenital hypothyroidism, dwarfism, and prolactin deficiency analogous to the phenotype of the *Snell* and *Jackson dwarf* (*dw*) mice (16). In contrast to *POU3F4*, *POU1F1* mutations are not confined to the bipartite DNA binding domain (14). Three mutations are located in the POU_s domain and one missense mutation has been described at a highly conserved position in the major transactivation domain (17). The clustering of missense mutations in the POU_h domain of *POU3F4* in patients with DFN3 suggests that either missense mutations at the aminoterminal half of the protein are not tolerated or, alternatively, are associated with another disease phenotype. Recently, it was shown that class III POU proteins can form heteroduplexes (18) which may explain the absence of missense mutations in the aminoterminal half and middle parts of the protein. Several observations suggest that this part of POU proteins may play an important role in the association of POU proteins with other transcription factors. A pivotal role for the POU_s domain in DNA dependent dimerization of Pit1 has been demonstrated (17). Moreover, activation of the α-internexin promoter by Brn-3a is dependent on the aminoterminal region of the protein (19), while an aminoterminal splice variant of Brn-3a has oncogenic potential (20). Apart from its expression in the developing inner ear, *POU3F4* is expressed in fetal brain and fetal kidney. The rat homologue of *POU3F4*, *RSH2*, is predominantly expressed in the fetal brain, in the otic vesicles, and in the outer sheath cells of the whisker roots (9), while the mouse homologue *Pou3f4* is expressed in the developing and adult central nervous system (21,22), and the pituitary gland (23). The *Xenopus*

homologue of *POU3F4*, *XlPOU2*, is expressed in the fetal head and the adult kidney and brain (24). Several studies concentrating on the role of the *POU3F4* homologues in the developing brain have suggested that *POU3F4* has, along with other members of its class, a pivotal role in neurogenesis (e.g. 24,25). Likewise, both *Brn.3-1* and *Brn.3-2* show a prominent expression throughout the nervous system. This raises the question why the pathological changes associated with mutations in these POU genes are confined to the inner ear or retina, respectively. Several mechanisms may account for this phenomenon. Proper spatial and temporal function of these POU proteins in the affected tissues may depend on the presence of other antagonistic POU domain containing regulators, resulting in a transcriptional imbalance of the downstream cascade (26). Alternatively, interaction with other tissue-specific co-factors may be required for proper functioning as has been demonstrated for the B cell-specific octamer coactivator *BOB.1* (*OBF.1*) which allows *Oct1* and *Oct2* to interact with octamer motifs upstream of promotors (27-29). Finally, the restricted pathological changes may also be due to a functional redundancy of these POU domain proteins in the non-affected tissues (30). However, the apparent lack of missense mutations outside the POU_h domain may be indicative for *POU3F4* functions other than those involving inner ear development.

Ten DFN3 patients have been identified so far with deletions upstream from *POU3F4* (31). These deletions range between 15kb and 1200kb proximal to the *POU3F4* gene, and share a small deleted region approximately 900kb proximal to the *POU3F4* gene.

Several mechanisms can explain the association of these deletions with DFN3. Deletions upstream of *POU3F4* may disrupt a yet unidentified exon of the *POU3F4* gene. This would mean that *POU3F4* contains an unusually large intron of 900kb in its 5' untranslated region. Although exceptional, very large introns have been described before, e.g. for the dystrophin gene where exon 1 of the dystrophin variant expressed in brain is located 400kb proximal from exon 2 (32). DNAse I footprinting studies have revealed the presence of several *Pou3f4* binding sites in a region up to 500bp upstream of the *Pou3f4* transcriptional start site (18). Although it is difficult to compare these *in vitro* results with the *in vivo* transcription mechanisms, they are suggestive for autoregulation, a common mechanism for POU genes. Moreover, they make the presence of a promotor several hundred kb centromeric less likely. Alternatively, silencing of the *POU3F4* gene in the larger deletions may be the result of a position effect that juxtaposes heterochromatic sequences near the *POU3F4* gene (*chapter 2.2*). For the *PAX6* gene, another transcription factor for which translocations have been described upstream from the gene in patients with aniridia (33-35), a fixed position with respect to the scaffold was recently demonstrated in extracted metaphase chromosomes (36). The chromosomal scaffold is the residual proteinaceous structure that retains when chromosomes are depleted of histones and important functional elements such as centromeres, replication origins, telomeres, and transcription sites may be

associated with this scaffold (37-39) Because *PAX6* was not expressed in the cell line of investigation, it was not possible to show that transcription of this gene depends on its fixed position with respect to the mitotic scaffold Accordingly, it will be interesting to study the spatial relationship between *POU3F4* and the mitotic scaffold in normal X chromosomes and in chromosomes from DFN3 patients with deletions upstream from *POU3F4*, particularly in cells in which *POU3F4* is expressed

Transcription of the *POU3F4* gene may also be due to disruption of promotor or enhancer sequences upstream to the gene The recently identified *Pou3f4* binding sites do not exclude the presence of additional binding sites farther upstream (18) There is significant evidence now that the locus control region of the β globin locus acts by creating domains in which promotor activity is permitted, i e preventing the formation of transcriptional repressive chromatin structures rather than regulating the rate of transcription (40,41) This mechanism may also apply for enhancers in general, and absence of similar transcriptional regulators in DFN3 patients with deletions upstream from *POU3F4* may keep the chromatin structure around the *POU3F4* gene in a repressed state Finally, another gene involved in inner ear development may be located in this genomic segment, although this seems less likely

Recently, coexpression of several class III POU proteins (Brn-1, Brn-2, Brn-4, and SCIP) during the formation of the mouse hypothalamic-pituitary axis was reported (23) *Brn2*$^{-/-}$ mice show a normal initial hypothalamic development followed by a failure of differentiation and maturation of specific hypothalamic neurosecretory neurons (23,42,43) The spatially and temporally overlapping expression patterns of Brn-2 and Brn-4, and the critical role for Brn-2 in late in the development of neuronal tissues, suggest a functional redundancy between Brn-4 and Brn-2 during the initial phases of neurogenesis (23) On the other hand, it has been demonstrated that *RHS2* activates the gonadotropin releasing hormone (*GnRH*) promotor activity in a GnRH-secreting hypothalamic neuron cell line, whereas SCIP, another POU domain protein, represses *GnRH* promotor activity (44) Thus, these POU proteins exert antagonistic effects upon GnRH release Similar observations have been made for Oct-1 The POU protein Oct-1 binds to the *GnRH* enhancer *in vitro*, and its binding is critical for the transcriptional activity of this enhancer in GnRH-secreting hypothalamic neuron cells (45) GnRH is a neuropeptide secreted by the hypothalamus which stimulates the excretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by the anterior pituitary In turn, LH stimulates testicular Leydig cells to produce testosterone Against this background, it is remarkable that hypogonadism has been reported as one of the hitherto unexplained additional clinical features in affected family members with DFN3 and a deletion of the *POU3F4* gene (46)

X,a translocations with chromosomal breakpoints in the Xq13-q21 region are frequently associated with gonadal dysfunction which suggests a critical role of this region in ovarian development (47) However, recent studies shed doubt on the presence of genes for ovarian development on the X chromosome, and

suggest that gonadal dysgenesis in females with sex chromosome rearrangements is primarily attributable to failure of chromosome pairing during meiotic prophase (48) The apparent clustering in Xq13-q21 may thus be related to the particularly late replication of the Xq21 band causing delayed or incomplete synapse formation Still, in view of the possible sensitivity of *POU3F4* transcription to its chromosomal environment (*chapter 7*), and its putative role in GnRH release, it is tempting to speculate that *POU3F4* contributes to the gonadal dysgenesis seen in patients with rearrangements of the Xq13-q21 segment The generation of *Brn4* deficient mice by targeted inactivation of the *Pou3f4* gene will further clarify the role of this POU protein in mammalian development

8.2 X-Linked Mental Retardation: Genetic and Molecular Dissection of a Common Heterogeneous Disease

Population studies of MR have suggested that X-linked genes account for approximately 20-50% of all cases with severe MR as judged from the higher prevalence of MR in males than in females It has been estimated that at least 7 to 15 X chromosomal genes play a major role in MR (49-54) At present, almost 150 X-linked syndromes have been reported in which MR is one of the clinical features (55)

The most prevalent XLMR syndrome accounting for approximately 30% of all cases with XLMR is the fragile X syndrome (FRAXA) The FRAXA syndrome is associated with a cytogenetically recognizable folate-sensitive fragile site and the physical fine-mapping of this fragile site was instrumental for the positional cloning of the fragile X mental retardation (*FMR-1*) gene (56) The 5' non-coding region of the *FMR-1* gene contains a CGG repeat which is unstable in FRAXA-families and shows massive expansion in affected individuals The gradual expansion of the repeat length in subsequent generations of FRAXA-families explains the phenomenon of genetic anticipation seen in FRAXA families, i e the increasing severity, or decreasing age of onset in successive generations (57) This new concept of repeat expansion soon led to the identification of several other genes containing unstable trinucleotide repeats in patients with neuromuscular disorders and MR (reviewed in 57,58) For another, albeit non-syndromic form of XLMR associated with a second closely linked fragile site on the X chromosome, FRAXE (59), a candidate gene (*FMR2*) has recently been cloned (60-62) Progress has also been made in the identification of genes involved in syndromic forms of XLMR One recent success was the identification of mutations in the *XH2* gene in patients with the clinically well-defined ATR-X syndrome which includes MR and α-thalassaemia (63,64) Mutations in the *XH2* gene have also been found in a patient previously diagnosed with Coffin-Lowry syndrome symptoms, patients with the Juberg-Marsidi MR syndrome, and in other mentally retarded

individuals without α-thalassaemia. Therefore, it is conceivable that the *XH2* gene plays an important role in MR-dysmorphy syndromes (65-67).

Despite these findings, the majority of the disorders giving rise to XLMR have not yet been elucidated at the molecular level. Particularly, this holds true for non-syndromic XLMR. Here, linkage analysis is confined to individual families due to heterogeneity of the disorder. Although these studies have revealed the presence of at least eight different gene loci that play a role in non-specific XLMR (68), the linkage intervals are still too wide to allow reliable genetic counseling in these families and the identification of the causative gene defects by positional cloning and related strategies. For the genetic elucidation of non syndromic forms of MR, the molecular analysis of MR-associated chromosomal rearrangements may be without alternative. Indeed, this association has been recognized for a long time. Chromosomal abnormalities account for 40% of the severe and 10-20% of the mild forms of MR, and already in 1972, Breg *et al.* reported an elevated incidence of balanced translocations in mentally retarded individuals, followed by other reports that supported the association of balanced chromosome rearrangements with MR (69-71). Moreover, the recent observation that there is a clustering of MR-associated breakpoints at 50 chromosomal sites in the human genome further illustrates the potential of this approach for the elucidation of genes for human brain function (N. Tommerup, personal communication).

Recently, a systematic search for subchromosomal rearrangements in 99 mentally retarded individuals resulted in the identification of 3 small subtelomeric deletions (72). Taking into account the limited informativity of the DNA probes, their physical distance from the telomeres, and the fact that only 20 subtelomeric regions were studied, it was calculated that cryptic subtelomeric deletions may be present in at least 6% of the mentally retarded (72). With respect to XLMR, the recent identification of two microdeletions on the short arm of the X chromosome in a familiar and sporadic case with MR, should enable the cloning of the underlying gene defects (73,74).

During this study we have focused on the mapping and cloning of genes for XLMR by analyzing MR-associated X chromosomal rearrangements. In a large panel of patients with MR, CHM, and/or DFN3 numerous deletions of the Xq21 band have been identified (75). The molecular characterization of these deletions has been the basis for the cloning of the genes underlying CHM (76-78) and DFN3 (*chapter 6*). Until recently, MR had only been described in patients carrying large deletions of the Xq21 region including the genes for DFN3 and CHM. The existence of a locus for non-syndromic MR between DFN3 and CHM was based on the identification of a small chromosomal segment that was deleted in patients with complex phenotypes including MR carrying large X chromosomal deletions encompassing the *POU3F4* and *CHM* genes, but present in all DFN3 and CHM patients with deletions spanning the *POU3F4* or *CHM* gene, respectively. Therefore, the identification of deletions in Xq21 in two patients with MR and CHM (*chapter 4*, 79) substantiates the presence of a non-syndromic MR locus between DFN3 and CHM. Surprisingly, deletions of the

Xq21 band have not been reported so far in patients with non-syndromic MR, nor in patients with DFN3 and MR Also, screening of a limited number of patients with MR and patients with MR and DFN3, has not resulted in the identification of deletions in Xq21 The failure to detect Xq21 deletions in patients with non-syndromic XLMR may be explained by the considerable genetic heterogeneity of the disorder In contrast, the apparent absence of Xq21 deletions in patients with DFN3 and MR remains enigmatic So far, three patients with deletions in Xq21 associated with MR and CHM have been reported (MBU, TM and AP (79,80, *chapter 4*)) Deletion analysis enabled us to map the MR gene between *POU3F4* and DXS121 while others (79) have argued that the MR gene must be located farther distal based on deletion studies in an additional patient with CHM and MR However, the mild MR in one patient carrying a microdeletion associated with DFN3 (81), and whose deletion defines the proximal boundary of the MR locus, may favour our more proximal position of the MR locus These authors proposed *ZNF6*, a zinc finger gene thought to function as a transcriptional regulator (82), as a candidate gene for MR in Xq21 on the basis of its localization between DXS326 and *CHM* and its high expression in the brain (79) *ZNF6* is a member of a large family of zinc finger genes, and gene defects in *ZNF6* may be masked by an autosomal homologue taking over its function If gene defects in *ZNF6* indeed result in MR, then this mechanism may explain the apparent discrepancy in the localization of the MR gene A similar situation applies to the *CHM* gene where a patient with a large Xq21 deletion including *CHM* has been described who has no clinical symptoms of CHM (83) Here, the autosomal homologue for *CHM*, *CHML* or *REP-2*, may be able to compensate for the absence of *CHM*-features in the patient (84) Complementation by a related gene may also explain the absence of deafness in another patient who carries a large deletion including the *POU3F4* gene (85)

Recently, a cyclophilin gene-like sequence has been mapped to the Xq21 1 region (86) The cyclophilin multigene family comprises of different members, several of which are still uncharacterized, and may play a role in the correct folding of specific protein substrates (87) Precise mapping of and sequence analysis of this putative gene should reveal whether it plays a role in MR Other candidate genes should soon be identified now that the YAC contig of this region has been completed

Mapping studies have been reported for 42 families with non-syndromic XLMR and an MRX number has been assigned to these families (55) These studies indicate that the Xq24-q25 band seems to be relatively devoid of XLMR genes while the clustering of linkage intervals in Xp proximal is particularly evident (68) In fact, almost half of the families show linkage to the Xp11 2-p21 1 region which may be indicative for either the presence of one particularly important gene for XLMR, or the presence of several genes that play a role in XLMR However, for all of these families but one, in which screening with microsatellite markers enabled the identification of an 1 Mb microdeletion that

cosegregates with the disorder (73), the intervals of linkage are still too wide to allow genetic counselling or the cloning of the underlying gene defect

To search for candidate genes for XLMR, we have set out to characterize X chromosomal breakpoints in mentally retarded females with balanced X,a translocations We have collected four of these MR-associated X,a translocations and mapped the X chromosomal breakpoints to distinct loci on the X chromosome YAC fluorescence *in situ* hybridization on metaphase chromosomes of these patients has enabled us to identify YACs for each X chromosomal breakpoint in these translocations Cytogenetically, three of these breakpoints had been assigned to the same band, Xp11, which harbours the defect in almost half of the MRX families studied These data may indicate that there is a common, particularly important XLMR gene in this region, or alternatively, that several XLMR genes may be clustered in this chromosomal segment We have shown that these breakpoints do not colocalize, but rather map to distinct X chromosomal regions Therefore, our mapping data suggest that the clustering of mapping intervals is more likely due to the presence of several genes for XLMR, than to one predominant gene

Although a priori, it is much more likely that in X,a translocations aberrant phenotypes are due to disruption or inactivation of X linked genes (*chapter 2 3*), a contribution of the autosomal breakpoints cannot be ruled out Therefore, we have also examined the autosomal breakpoint regions in the above mentioned translocations In all patients, the direct involvement of obvious autosomal candidate genes could be excluded, i e involvement of the α4 subunit of the nicotinic acetylcholine receptor gene (*CHRNA4*) implied in several autosomal dominant forms of epilepsy (88,89), of the small nuclear ribonucleoprotein polypeptide N gene (*SNRPN*) mapped to the Prader-Willy syndrome (PWS) region (90,91), and of the lissencephaly-1 (*LIS1*) gene (92) encoding the 45 kDa subunit of the platelet-activating factor acetylhydrolase (93) which has recently been excluded from beeing involved in Miller-Dieker lissencephaly syndrome (94,95) Since imprinting in the PWS region is not confined to the *SNRPN* locus, but extends in both directions, we subsequently analyzed the entire YAC contig extending into the proximal deletion breakpoint region (96,97) Fluorescence *in situ* hybridization of these YACs to metaphase chromosomes of the patient revealed that the proximal deletion breakpoint region in PWS patients is located distally from the breakpoint This makes the involvement of the imprinted region on chromosome 15 in the clinical phenotype of the patient less likely However, the derivative X chromosomes carrying the relevant autosomal genes, also harbour the XIC Therefore, it is conceivable that the observed phenotype is influenced by the variable spreading of X inactivation into the autosomal segment in a small subset of cells The precise assignment of the breakpoints with respect to these genes should help us to clarify this issue

The fourth translocation maps to Xq13 1 and has been studied in more detail The molecular analysis of this X chromosomal breakpoint has led to the identification of an alternatively spliced X-linked gene, *DXS6673E*, of which

the 5' untranslated region is disrupted by the translocation (*chapter 9*) Apart from MR, the only conspicuous findings in this patient are scoliosis and spotty abdominal hypopigmentation of the skin (98) Since the *DXS6673E* gene underlies X-inactivation, and replication studies showed a consistently late replicating normal X chromosome, we expected that in the patient this gene would not be expressed Surprisingly however, when using primers specific for both splice variants of *DXS6673E*, normal gene products were generated by RT-PCR on RNA from the patient It could be demonstrated that one of the splice variants is not disrupted by the breakpoint and that a second variant encoding the same protein is most likely controlled by sequences from the translocated chromosome 13 This finding does not rule out the possibility that the abnormal phenotype seen in this patient results from aberrant spatial or temporal gene expression, but this hypothesis is difficult to prove A similar situation has been reported for translocations disrupting two variants of the T-cell translocation gene *TTG-2* in acute T cell leukemias where both variants encode the same protein and are under control of different promotors (99)

In spite of the clear evidence for disruption of the *DXS6673E* gene, the possibility that genes on chromosome 13 contribute to the observed phenotype of the patient can not be excluded Several patients have been reported with large deletions of the long arm of chromosome 13 (13q syndrome) including band 13q31 and resulting in a partial monosomy for chromosome 13 (100-102) The translocation may thus disrupt a dosage dependent gene on chromosome 13, or, again, spreading of X-inactivation into the autosomal segment of the derivative chromosome 13 in a small subset of cells may silence dosage dependent genes Cloning of the breakpoint region on chromosome 13 should soon clarify the possible role of other genes, or their promotor regions, in the aetiology of MR in this patient

8.3 Outlook

As pointed out in the introduction, the elucidation of gene defects underlying multifactorial disorders will be of extreme importance in the years to come In fact, most of the common disorders such as diabetes mellitus or hypertension and most of the congenital malformations (e g cleft lip and palate or neural tube defects) are not monogenic In polygenic disorders, many genetic factors (quantitative trait loci, QTL) contribute cumulatively to the disease phenotype, whereas in multifactorial disorders also environmental factors play an important role Different approaches like linkage analysis, allele sharing methods, and association studies are at our disposal to unravel these disorders (103) The recent successes in the identification of a schizophrenia susceptibility locus on chromosome 6p by several groups demonstrates the application of linkage analysis for multifactorial traits (104-106) For another multifactorial disorder, diabetes, much progress has been made Both allele sharing methods (103,107), as well as other strategies (108-111) have been

applied succesfully to dissect diabetes genetically. Diabetes research has now focused on the positional cloning of genes by whole genome searches along with specific testing of candidate genes, particularly in areas of positive linkage. Against this background, balanced and unbalanced chromosome aberrations that are associated with multifactorial diseases as diabetes may also contribute to the identification of important gene defects. Eventually, mutation analysis of disease-associated candidate genes in large populations should reveal the true value of this approach.

8.4 References

1 Verrijzer, C P and van der Vliet, P C (1993) POU domain transcription factors. *Biochim Biophys Acta*, **1173**, 1-21.

2 Wright, P E. (1994) POU domains and homeodomains *Curr Biol*, **4**, 22-27.

3 Assa-Munt, N., Mortishire-Smith, R.J., Aurora, R., Herr, W., and Wright, P E. (1993) The solution structure of the Oct-1 POU-specific domain reveals a striking similarity to the bacteriophage lambda repressor DNA-binding domain. *Cell*, **73**, 193-205

4 Dekker, N., Cox, M., Boelens, R , Verrijzer, C.P., van der Vliet, P C , Kaptein, and R. (1993) Solution structure of the POU-specific DNA-binding domain of Oct- *Nature*, **362**, 852-855.

5 Ryan, A.F , Crenshaw, E.B , III, and Simmons, D.M (1991) Gene expression in normal and abnormal inner ears *Ann NY Acad Sci*, **630**, 129-132.

6 Erkman, L., McEvilly, R.J , Luo, L , Ryan, A K , Hooshmand, F , O'Connell, S M , Keithley, E.M., Rapaport, D H , Ryan, A F , and Rosenfeld, M G (1996) Role of transcription factors Brn-3 1 and Brn-3.2 in auditory and visual system development *Nature*, **381**, 603-606

7 Gan, L , Xiang, M.Q , Zhou, L J., Wagner, D S , Klein, W H , and Nathans, J (1996) POU domain factor Brn-3B is required for development of a large set of retinal ganglion cells *Proc Natl Acad Sci USA*, **93**, 3920-3925

8 Douville, P J , Atanasoski, S , Tobler, A., Fontana, A , and Schwab, M E. (1994) The brain-specific POU-box gene *Brn4* is a sex-linked transcription factor located on the human and mouse X chromosomes. *Mamm Genome*, **5**, 180-182

9 le Moine, C. and Young, W S , III. (1992) *RHS2*, a POU domain-containing gene, and its expression in developing and adult rat. *Proc Natl Acad Sci USA*, **89**, 3285-3289.

10. Bitner-Glindzicz, M., Turnpenny, P., Höglund, P., Kääriäinen, H , Sankila, E , van der Maarel, S M , de Kok, Y.J.M., Ropers, H., Cremers, F P.M., Pembrey, M , and Malcolm, S. (1995) Further mutations in *brain 4* clarify the phenotype in the X-linked deafness, DFN3. *Hum Mol Genet*, **4**, 1467-1469

11 de Kok YJM, Cremers CWRJ, Ropers H-H, Cremers FPM Somatic mosaicism for a *POU3F4* missense mutation in a patient with X-linked deafness type 3 (DFN3) submitted

12. Radovick, S., Nations, M., Du, Y , Berg, L A , Weintraub, B.D., and Wondisford, F.E (1992) A mutation in the POU-homeodomain of Pit-1 responsible for combined pituitary hormone deficiency *Science*, **257**, 1115-1118

13 Pfaffle, R W , DiMattia, G E , Parks, J S , Brown, M R , Wit, J M , Jansen, M , van der Nat, H , van den Brande, J L , Rosenfeld, M G , and Ingraham, H A (1992) Mutation of the POU-specific domain of Pit-1 and hypopituitarism without pituitary hypoplasia *Science*, **257**, 1118-1121.

14 Ohta, K , Nobukuni, Y., Mitsubuchi, H , Fujimoto, S., Matsuo, N , Inagaki, H., Endo, F , and Matsuda, I (1992) Mutations in the Pit-1 gene in children with combined pituitary hormone deficiency. *Biochem Bioph Res Co.*, **189**, 851-855.

15 Tatsumi, K., Miyai, K., Notomi, T., Kaibe, K., Amino, N , Mizuno, Y , and Kohno, H (1992) Cretinism with combined hormone deficiency caused by a mutation in the PIT1 gene *Nature Genet* , **1**, 56-58

16 Li, S , Crenshaw, E.B , 3d, Rawson, E.J., Simmons, D M , Swanson, L W., Rosenfeld, and MG (1990) Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene pit-1. *Nature*, **347**, 528-533

17. Ingraham, H A , Flynn, S.E., Voss, J.W., Albert, V.R , Kapiloff, M S., Wilson, L , and Rosenfeld, M.G (1990) The POU-specific domain of Pit-1 is essential for sequence-specific, high affinity DNA binding and DNA-dependent Pit-1-Pit-1 interactions. *Cell*, **61**, 1021-1033

18 Malik, K.F., Kim, J., Hartman, A.L., Kim, P , and Young, W S. (1996) Binding preferences of the POU domain protein Brain-4: implications for autoregulation *Molec Brain Res* , **38**, 209-221

19. Budhram-Mahadeo, V., Morris, P J., Lakin, N.D., Theil, T , Ching, G.Y., Lillycrop, KA, Moroy, T., Liem, R.K., and Latchman, D.S. (1995) Activation of the alpha-internexin promoter by the Brn-3a transcription factor is dependent on the N-terminal region of the protein *J Biol Chem* , **270**, 2853-2858.

20 Theil, T , McLean-Hunter, S , Zornig, M , and Moroy, T. (1993) Mouse Brn-3 family of POU transcription factors: a new aminoterminal domain is crucial for the oncogenic activity of Brn-3a *Nucleic Acids Res* , **21**, 5921-5929.

21. Hara, Y , Rovescalli, A C , Kim, Y., and Nirenberg, M (1992) Structure and evolution of four POU domain genes expressed in mouse brain *Proc Natl Acad Sci USA* , **89**, 3280-3284.

22 Mathis, J M , Simmons, D M , He, X , Swanson, L W , and Rosenfeld, M G. (1992) Brain 4 a novel mammalian POU domain transcription factor exhibiting restricted brain-specific expression *EMBO Journal*, **11**, 2551-2561

23. Schonemann, M.D., Ryan, A.K., McEvilly, R.J., O'Connell, S.M., Arias, C.A., Kalla, KA, Li, P , Sawchenko, P.E , and Rosenfeld, M G (1995) Development and survival of the endocrine hypothalamus and posterior pituitary gland requires the neuronal POU domain factor Brn-2. *Gene Dev*, **9**, 3122-3135.

24 Agarwal, V.R. and Sato, S.M (1991) XLPOU 1 and XLPOU 2, two novel POU domain genes expressed in the dorsoanterior region of Xenopus embryos *Dev Biol* , **147**, 363-373.

25 Alvarez-Bolado, G , Rosenfeld, M.G., and Swanson, L W (1995) Model of forebrain regionalization based on spatiotemporal patterns of POU-III homeobox gene expression, birthdates, and morphological features *J Comp Neurol* , **355**, 237-295

26. Sasaki, H., Yamamoto, M , and Kuroiwa, A. (1992) Cell type dependent transcription regulation by chick homeodomain proteins *Mech Dev* , **37**, 25-36.

27 Strubin, M., Newell, J.W., and Matthias, P (1995) OBF-1, a novel B cell-specific coactivator that stimulates immunoglobulin promoter activity through association with octamer-binding proteins *Cell*, **80**, 497-506.

28 Gstaiger, M., Knoepfel, L , Georgiev, O., Schaffner, W., and Hovens, C M (1995) A B-cell coactivator of octamer-binding transcription factors *Nature*, **373**, 360-362.

29 Pfisterer, P , Zwilling, S , Hess, J , and Wirth, T (1995) Functional characterization of the murine homolog of the B cell-specific coactivator BOB 1/OBF 1 *J Biol Chem* , **270**, 29870-29880

30 Erickson, H P (1993) Gene knockouts of c-src, transforming growth factor beta 1, and tenascin suggest superfluous, nonfunctional expression of proteins *J Cell Biol* , **120**, 1079-1081

31 de Kok, Y J M , Vossenaar, E R , Cremers, C W R J , Dahl, N , Laporte, J , Hu, L -J , Lacombe, D , Fischel-Ghodsian, N , Friedman, R A , Parnes, L A , Thorpe, P , Bitner-Glindzicz, M , Pander, H -J , Heilbronner, H , Graveline, J , den Dunnen, J T , Brunner, H G , Ropers, H H , and Cremers, F P M (1996) Identification of a hot spot for microdeletions in patients with X-linked deafness type 3 (DFN3) 900 kb proximal to the DFN3 gene *POU3F4* *Hum Mol Genet* , **5**, 1229-1235

32 Boyce, F M , Beggs, A H , Feener, C , and Kunkel, A M (1991) Dystrophin is transcribed in brain from a distant upstream promotor *Proc Natl Acad Sci USA* , **88**, 1276-1280

33 Gessler, M , Simola, K O J , and Bruns, G A P (1989) Cloning of breakpoints of a chromosome translocation identifies the AN2 locus *Science*, **244**, 1575 1578

34 Ton, C C T , Hirvonen, H , Miwa, H , Weil, M M , Monaghan, P , Jordan, T , van Heyningen, V , Hastie, N D , Meijers-Heijboer, H , Drechsler, M , Royer-Pokora, B , Collins, F , Swaroop, A , Strong, L C , and Saunders, G F (1991) Positional cloning and characterization of a paired box- and homeobox-containing gene from the aniridia region *Cell*, **67**, 1059-1074

35 Jordan, T , Hanson, I , Zaletayev, D , Hodgson, S , Prosser, J , Seawright, A , Hastie, N , and van Heyningen, V (1992) The human PAX6 gene is mutated in two patients with aniridia *Nature Genet* , **1**, 328-332

36 Bickmore, W A and Oghene, K (1996) Visualizing the spatial relationships between defined DNA sequences and the axial region of extracted metaphase chromosomes *Cell*, **84** 95-104

37 Gasser, S M , Amati, B B , Cardonas, M E , and Hofmann J F -X (1989) Studies on scaffold attachment sites and their relation to genome function *Int Rev Cytol* , **119**, 57-96

38 Razin, S V , Hancock, R , Iarovaia, O , Westergaard, O Gromova, I , Georgiev and GP (1993) Structural-functional organization of chromosomal DNA domains *Cold Spring Harbor Symposia on Quantitative Biology*, **58**, 25-35

39 Cook, P R (1994) RNA polymerase structural determinant of the chromatin loop and the chromosome *Bioessays*, **16**, 425-430

40 Walters, M C , Magis, W , Fiering, S , Eidemiller, J , Scalzo, D , Groudine, M , and Martin, D I (1996) Transcriptional enhancers act in cis to suppress position-effect variegation *Gene Dev* , **10**, 185 195

41 Martin, D I K , Fiering, S , and Groudine, M (1996) Regulation of b-globin gene expression straightening out the locus *Curr Opin Genet Dev* , **6**, 488 495

42 Nakai, S , Kawano, H , Yudate, T , Nishi, M , Kuno, J , Nagata, A , Jishage, K Hamada, H , Fujii, H , Kawamura, K , Shiba, K , Noda, T , Pou transcription factor, Brn 2, Neuronal development, Magnocellular, neurons, Paraventricular nucleus, and Supraoptic nucleus (1995) The

POU domain transcription factor Brn-2 is required for the determination of specific neuronal lineages in the hypothalamus of the mouse *Gene Dev*, **9**, 3109-3121.

43 Sharp, Z D and Morgan, W W (1996) Brain POU-er *Bioessays*, **18**, 347-350.

44. Kepa, J K, Jacobsen, B M, Bruder, J.M and Wierman, M E (1995) Brain-4 (Brn-4) pou protein activates rat GnRH promoter activity in GT1-7 neuronal cells and blocks repression by SCIP *77th annual meeting of the Endocrine Society*, 53.

45. Clark, M E. and Mellon, P.L (1995) The POU homeodomain transcription factor Oct-1 is essential for activity of the gonadotropin-releasing hormone neuron-specific enhancer. *Mol Cell Biol*, **15**, 6169-6177.

46 Bach, I, Brunner, H G, Beighton, P., Ruvalcaba, R H A, Reardon, W, Pembrey, M E., van der Velde-Visser, S D, Bruns, G A P, Cremers, C.W R J, Cremers, F P M., and Ropers, H. (1992) Microdeletions in patients with gusher-associated, X-linked mixed deafness (DFN3) *Am J Hum Genet*, **50**, 38-44

47 Therman, E and Susman, B. (1990) The similarity of phenotypic effects caused by Xp and Xq deletions in the human female: a hypothesis *Hum Genet*, **85**, 175-183

48 Ogata, T and Matsuo, N (1996) Turner syndrome and female sex chromosome aberrations: deduction of the principal factor involved in the development of clinical features. *Hum Genet.*, **95**, 607-629

49 Turner, G and Turner, B. (1974) X-linked mental retardation *J Med Genet*, **11**, 109-113

50 Herbst, D S and Miller, J.R. (1980) Nonspecific X-linked mental retardation II the frequency in British Columbia *Am J Med Genet*, 7, 461-469

51 Opitz, J.M. (1986) Editorial comment on the gates of hell and a most unusual gene. *Am J Med Genet.*, **23**, 1-10.

52 Opitz, J.M (1987) Erratum. *Am J Med Genet*, **26**, 37.

53 Kerr, B., Turner, G., Mulley, J, Gedeon, A, and Partington, M. (1991) Non-specific X-linked mental retardation *J Med Genet*, **28**, 378-382

54 Glass, I.A (1991) X linked mental retardation *J Med Genet*, **28**, 361-371

55 Lubs, H A., Chiurazzi, P, Arena, J F, Schwartz, C, Tranebjaerg, L, and Neri, G. (1996) XLMR genes: update 1996. *Am J Med Genet*, **64**, 147-157.

56 Verkerk, A.J.M.H., Pieretti, M., Sutcliffe, J.S, Fu, Y., Kuhl, D P.A., Pizzuti, A., Reiner, O, Richards, S, Victoria, M F, Zhang, F., Eussen, B.E, van Ommen, G.B, Blonden, L.A J., Riggins, G J., Chastain, J L., Kunst, C.B, Galjaard, H, Caskey, C T., Nelson, D L, Oostra, B.A, and Warren, S T. (1991) Identification of a gene (*FMR-1*) containing a CGG repeat coincident with a fragile X breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell*, **65**, 905-914.

57 Ashley, C T.Jr., Warren, S.T, Trinucleotide repeat, Genome instability, Genetic anticipation, Mental, retardation, Neurodegenerative disease, Dynamic mutations, Fragile, and sites (1995) Trinucleotide repeat expansion and human disease *Annu Rev Genet*, **29**, 703-728

58 Willems, P.J. (1994) Dynamic mutations hit double figures *Nature Genet*, **8**, 213-215.

59 Knight, S.J L, Flannery, A V, Hirst, M C., Campbell, L, Christodoulou, Z., Phelps, S.R, Pointon, J, Middleton-Price, H R., Barnicoat, A., Pembrey, M E, Holland, J, Oostra, B A, Bobrow, M, and Davies, K E (1993) Trinucleotide repeat amplification and hypermethylation of a CpG island in *FRAXE* mental retardation *Cell*, **74**, 127-134.

60. Chakrabarti, L., Knight, S.J.L, Flannery, A.V, and Davies, K.E. (1996) A candidate gene for mild mental handicap at the FRAXE fragile site. *Hum Mol. Genet.*, **5**, 275-282.

61 Gecz, J., Gedeon, A K, Sutherland, G R, and Mulley, J C. (1996) Identification of the gene *FMR2*, associated with *FRAXE* mental retardation. *Nature Genet*, **13**, 105-108.

62. Gu, Y., Shen, Y, Gibbs, R.A., and Nelson, D.L. (1996) Identification of *FMR2*, a novel gene associated with the *FRAXE* CCG repeat and CpG island *Nature Genet*, **13**, 109-113

63 Gibbons, R.J, Picketts, D J., Villard, L, and Higgs, D.R. (1995) Mutations in a putative global transcriptional regulator cause X-linked mental retardation with a-thalassemia (ATR-X syndrome) *Cell*, **80**, 837-845

64 Gibbons, R J, Picketts, D J, and Higgs, D.R. (1995) Syndromal mental retardation due to mutations in a regulator of gene expression *Hum Mol Genet*, **4**, 1705-1709

65. Villard, L, Toutain, A, Moraine, C and Fontes, M. (1996) XNP a gene coding for a potential global transcription factor, is involved in the ATRX and related MR syndromes *Am J Med Genet*,(Abstract).

66 Villard, L., Gecz, J., Mattei, J.F, Fontes, M, Saugier-Veber, P, Munnich, A, and Lyonnet, S (1996) XNP mutation in a large family with Juberg-Marsidi syndrome *Nature Genet*, **12**, 359-360

67. Ion, A, Telvi, L., Chaussain, J L, Galacteros, F., Valayer, J., Tellous, M., and McElreavey, K (1996) A novel mutation in the putative DNA helicase *XH2* is responsible for male-to-female sex reversal associated with an atypical form of the ATR-X syndrome. *Am J Hum Genet*, **58**, 1185-1191.

68. Gedeon, A K., Donnelly, A J., Mulley, J C, Kerr, B, and Turner, G. (1996) How many X-linked genes for non-specific mental retardation (MRX) are there? *Am J Med Genet*, **64**, 158-162.

69. Breg, W.R., Miller, D.A, Allerdice, P.W, and Miller, O J (1972) Identification of translocation chromosomes by quinacrine fluorescence. *Am J Dis Child*, **123**, 561-564

70. Jacobs, P A. (1974) Correlation between euploid structural chromosome rearrangements and mental subnormality in humans *Nature*, **249**, 164-165.

71. Funderburk, S J, Spence, M.A, and Sparkes, R S (1977) Mental retardation associated with "balanced" chromosome rearrangements *Am. J Hum Genet*, **29**, 136-141.

72 Flint, J, Wilkie, A.O.M, Buckle, V J., Winter, R.M., Holland, A.J., and McDermid, H E (1995) The detection of subtelomeric chromosomal rearrangements in idiopathic mental retardation. *Nature Genet.*, **9**, 132-140.

73 Nonspecific mental retardation is probably caused by a microdeletion in a Belgian family. 1995, *Seventh international workshop on the Fragile X and X-linked mental retardation*

74 Billuart, P., Vinet, M C , des Portes, V , Llense, S , Richard, L , Moutard, M L , Recan, D , Bruls, T , Bienvenu, T., Kahn, A , Beldjord, C., and Chelly, J (1996) Identification by STS PCR screening of a microdeletion in Xp21 3-22 1 associated with non-specific mental retardation *Hum Mol Genet* , **5**, 977-979

75. Philippe, C , Arnould, C , Sloan, F., van Bokhoven, H , van der Velde-Visser, S.D., Chery, M , Ropers, H , Gilgenkrantz, S , Monaco, A P , and Cremers, F P M (1995) A high resolution interval map of the q21 region of the human X chromosome *Genomics*, **27**, 539-543

76 Cremers, F.P M., van de Pol, T.J R , van Kerkhoff, E P M , Wieringa, B , and Ropers, H (1990) Cloning of a gene that is rearranged in patients with choroideraemia *Nature*, **347**, 674-677.

77 Merry, D E , Jänne, P.A., Landers, J.E., Lewis, R.A , and Nussbaum, R.L (1992) Isolation of a candidate gene for choroideremia. *Proc Natl Acad Sci USA* , **89**, 2135-2139.

78 van Bokhoven, H , van den Hurk, J A J M , Bogerd, L , Philippe, C , Gilgenkrantz, S , de Jong, P., Ropers, H., and Cremers, F P M (1994) Cloning and characterization of the human choroideremia gene *Hum Mol Genet* , **3**, 1041-1046.

79 May, M , Colleaux, L., Murgia, A , Aylsworth, A., Nussbaum, R , Fontes, M , and Schwartz, C. (1995) Molecular analysis of four males with mental retardation and deletions of Xq21 places the putative MR region in Xq21 1 between DXS233 and CHM. *Hum Mol Genet* , **4**, 1465-1466.

80. Hodgson, S V., Robertson, M E , Fear, C N , Goodship, J , Malcolm, S , Jay, B , Bobrow, M , and Pembrey, M E (1987) Prenatal diagnosis of X-linked choroideremia with mental retardation, associated with a cytologically detectable X-chromosome deletion *Hum Genet* , **75**, 286-290

81 Myhre, S A , Ruvalcaba, R.H.A., and Kelley, V C. (1982) Congenital deafness and hypogonadism a new X-linked recessive disorder. *Clin Genet* , **22**, 299-307.

82 Lloyd, S L , Sargent, C.A , Chalmers, J , Lim, E , Habeebu, S S M , and Affara, N A (1991) An X-linked zinc finger gene mapping to Xq21 1-q21 3 closely related to ZFX and ZFY. possible origins from a common ancestral gene *Nucleic Acids Res* , **19**, 4835-4841.

83 Cremers, F.P.M , van de Pol, T J.R., Diergaarde, P J , Wieringa, B., Nussbaum, R L , Schwartz, M., and Ropers, H (1989) Physical fine mapping of the choroideremia locus using Xq21 deletions associated with complex syndromes. *Genomics*, **4**, 41-46

84 Cremers, F.P M , Armstrong, S.A , Seabra, M C., Brown, M.S., and Goldstein, J L. (1994) REP-2, a rab escort protein encoded by the choroideremia-like gene *J Biol Chem* , **269**, 2111-2117

85 Tabor, A , Andersen, O., Lundsteen, C., Niebuhr, E , and Sardemann, H (1983) Interstitial deletion in the "critical region" of the long arm of the X chromosome in a mentally retarded boy and his normal mother *Hum Genet* , **64**, 196-199.

86 Kandpal, G , Jacob, A N K , and Kandpal, R P (1996) A cyclophilin gene-like sequence maps to human X-chromosome *Somat Cell Mol Genet* , **22**, 67-73

87 Takahashi, N , Hayano, T., and Suzuki, M. (1989) Peptidyl-prolyl cis-trans isomerase is the cyclosporin A-binding protein cyclophilin *Nature* , **337**, 473-475.

88 Beck, C , Moulard, B , Steinlein, O , Guipponi, M., Valle, L , Montpied, P., Baldy-Moulinier, M , and Malafosse, A. (1994) A nonsense mutation in the a4 subunit of the nicotinic acetylcholine receptor (CHRNA4) cosegregates with 20q-linked benign neonatal familial convulsions (EBN1) *Neurobiol Dis* , **1**, 95-99

89 Steinlein, O K., Mulley, J C , Propping, P , Wallace, R H., Phillips, H A., Sutherland, G R., Scheffer, I.E , and Berkovic, S F (1995) A missense mutation in the neuronal nicotinic acetylcholine receptor alpha 4 subunit is associated with autosomal dominant nocturnal frontal lobe epilepsy *Nature Genet* , **11**, 201-203

90 Ozcelik, T , Leff, S., Robinson, W , Donlon, T , Lalande, M , Sanjines, E., Schinzel, A., and Francke, U. (1992) Small nuclear ribonucleoprotein polypeptide N (SNRPN), an expressed gene in the Prader-Willi syndrome critical region *Nature Genet* , **2**, 265-269.

91 Lalande, M (1994) In and around SNRPN. *Nature Genet* , **8**, 5-7

92. Reiner, O., Carrozzo, R , Shen, Y , Wehnert, M., Faustinella, F., Dobyns, W.B , Caskey, C.T., and Ledbetter, D.H. (1993) Isolation of a Miller-Dieker lissencephaly gene containing G protein beta-subunit-like repeats *Nature*, **364**, 717-721.

93. Hattori, M , Adachi, H., Tsujimoto, M., Arai, H., and Inoue, K. (1994) Miller-Dieker lissencephaly gene encodes a subunit of brain platelet-activating factor acetylhydrolase. *Nature*, **370**, 216-218

94 Chong, S.S , Pack, S., Tanigami, A , Carrozzo, R , Dobyns, W.B and Ledbetter, D H. (1995) Systematic deletion analysis of MDS and ILS patients excludes a candidate gene delineates the lissencephaly gene locus *Am J Hum Genet* , **57**, A34-(170) (Abstract)

95 Chong, S S., Tanigami, A , Roschke, A.V., and Ledbetter, D.H (1996) 14-3-3-ε has no homology to *LIS1* and lies telomeric to it on chromosome 17p13 3 outside the Miller-Dieker syndrome chromosome region *Genome Research*, **6**, 735-741

96. Mutirangura, A., Jayakumar, A , Sutcliffe, J S , Nakao, M , McKinney, M J., Buiting, K , Horsthemke, B., Beaudet, A.L , Chinault, A C , and Ledbetter, D H (1993) A complete YAC contig of the Prader-Willi/Angelman chromosome region (15q11-q13) and refined localization of the SNRPN gene. *Genomics*, **18**, 546-552.

97. Christian, S.L , Robinson, W.P , Huang, B , Mutirangura, A , Line, M.R., Nakao, M., Surti, U , Chakravarti, A , and Ledbetter, D.H (1995) Molecular characterization of two proximal deletion breakpoint regions in both Prader-Willi and Angelman syndrome patients *Am J Hum Genet* , **57**, 40-48.

98. Teboul, M., Mujica, P., Chery, M , Leotard, B., and Gilgenkrantz, S (1989) Translocations X-autosomes équilibrées et retard mental. Contribution à la cartographie des retards mentaux liés à L'X (à l'exclusion de L'X-FRA) *J. Génét Hum* , **37**, 179-195.

99. Royer-Pokora, B., Rogers, M., Zhu, T H , Schneider, S., Loos, U., and Bolitz, U (1995) The *TTG-2/RBTN2* T cell oncogene encodes two alternative transcripts from two promoters. the distal promoter is removed by most 11p13 translocations in acute T cell leukaemia's (T-ALL). *Oncogene*, **10**, 1353-1360

100 Allderdice, P W , Davis, J G , Miller, O.J , Klinger, H P., Warburton, D , Miller, DA, Allen, F H , Jr , Abrams, C.A , and McGilvray, E (1969) The 13q-deletion syndrome *Am J Hum Genet* , **21**, 499-512.

101 Brown, S., Gersen, S , Anyane-Yeboa, K , and Warburton, D (1993) Preliminary definition of a "critical region" of chromosome 13 in q32· report of 14 cases with 13q deletions and review of the literature. *Am J Med Genet* , **45**, 52-59

102 Brown, S., Russo, J., Chitayat, D , and Warburton, D. (1995) The 13q· syndrome the molecular definition of a critical deletion region in band 13q32 *Am J Hum Genet* , **57**, 859-866

103 Lander, E S and Schork, N J (1994) Genetic dissection of complex traits *Science*, **265**, 2037-2048.

104 Moises, H.W., Yang, L., Kristbjarnarson, H , Wiese, C , Byerley, W , Macciardi, F., Arolt, V., Blackwood, D., Liu, X , Sjögren, B , Aschauer, H.N , Hwu, H -G , Jang, K., Livesly, W.J., Kennedy, J L., Zoega, T , Ivarsson, O , Bui, M -T., Yu, M -H., Havsteen, B , Commenges, D., Weissenbach, J., Schwinger, E , Gottesman, I I., Pakstis, A J , Wetterberg, L., Kidd, K K., and Helgason, T. (1995) An international two-stage genome-wide search for schizophrenia susceptibility genes. *Nature Genet* , **11**, 321-324.

105. Schwab, S G , Albus, M , Hallmayer, J , Honig, S , Borrmann, M., Lichtermann, D., Ebstein, R P , Ackenheil, M , Lerer, B , Risch, N , Maier, W., and Wildenauer, D.B. (1995) Evaluation of a susceptibility gene for schizophrenia on chromosome 6p by multipoint affected sib-pair linkage analysis. *Nature Genet* , **11**, 325-327.

106 Mowry, B.J., Nancarrow, D.J., Lennon, D.P., Sandkuijl, L.A., Crowe, R R , Silverman, J M , Mohs, R C., Siever, L J., Endicott, J., Sharpe, L , Walters, M.K , Hayward, N K., Levinson, D F , Gurling, H , Kalsi, G , Hui-Sui Chen, A , Green, M , Butler, R , Read, T , Murphy, P., Curtis, D., Sharma, T., Petursson, H., Brynjolfsson, J., Antonarakis, S.E , Blouin, J -L , Pulver, A.E., Wolyniec, P., Lasseter, V K., Nestadt, G , Kasch, L., Babb, R , Kazazian, H H , Dombroski, B , Kimberland, M., Ott, J., Housman, D , Karayiorgou, M., and MacLean, C.J (1995) Schizophrenia susceptibility and chromosome 6p24-22. *Nature Genet* , **11**, 233-236

107. Hashimoto, L., Habita, C., Beressi, J.P., Delepine, M., Besse, C , Cambon-Thomsen, A., Deschamps, I., Rotter, J.I , Djoulah, S , James, M.R., Froguel, P , Weissenbach, J , Lathrop, G.M., and Julier, C. (1994) Genetic mapping of a susceptibility locus for insulin-dependent diabetes mellitus on chromosome 11q *Nature*, **371**, 161-164.

108 Todd, J A (1995) Genetic analysis of type 1 diabetes using whole genome approaches. *Proc Natl Acad Sci. USA* , **92**, 8560-8565.

109 Cordell, H J and Todd, J.A. (1995) Multifactorial inheritance in type 1 diabetes *Trends Genet* , **11**, 499-504.

110. Vionnet, N., Stoffel, M , Takeda, J., Yasuda, K , Bell, G.I., Zouali, H., Lesage, S, Velho, G., Iris, F., Passa, P., and et al. (1992) Nonsense mutation in the glucokinase gene causes early-onset non-insulin-dependent diabetes mellitus. *Nature*, **356**, 721-722.

111. Hager, J., Hansen, L., Vaisse, C., Vionnet, N., Philippi, A , Poller, W., Velho, G, Carcassi, C , Contu, L., Julier, C., and et al. (1995) A missense mutation in the glucagon receptor gene is associated with non-insulin-dependent diabetes mellitus *Nature Genet.*, **9**, 299-304

9. SUMMARY

The aim of this study was to identify and characterize the gene for both X-linked deafness type 3 (DFN3) and candidate genes for X-linked mental retardation by positional cloning strategies Therefore, we defined and fine-mapped the critical regions for DFN3 and non-syndromic XLMR in Xq21 (*chapters 4 and 5*) by the molecular characterization of patients carrying interstitial deletions of Xq21 with CHM, DFN3 or MR, or a combination of these disorders These studies confirmed the previously defined locus for MR and narrowed down the critical region for DFN3 The molecular characterization of the DFN3 critical region, the mapping of the mouse POU domain containing gene *Pou3f4* on the mouse X chromosomal segment homologous to the human Xq13-q21 region, and the expression of the rat homologue *RHS2* in the otic vesicle, resulted in the identification of mutations in the human homologue *POU3F4* in patients with DFN3 (*chapter 6*) However, some patients with DFN3 carry a normal *POU3F4* gene, and have deletions or complex rearrangements upstream of *POU3F4* One such patient carrying a paracentric inversion/duplication has been characterized in great detail (*chapter 7*) The association between these rearrangements and DFN3 may be explained by the presence of an additional exon or regulatory sequences for *POU3F4*, a position effect interfering with proper *POU3F4* expression, or the presence of a second gene involved in inner ear development in this chromosomal segment

Additionally, we have fine-mapped the X chromosomal breakpoints of four balanced X,a translocations in mentally retarded females by fluorescence *in situ* hybridization Due to the preferential inactivation of the normal X chromosome in cells of these patients, these rearrangements may result in functional nullisomies for X chromosomal genes disrupted by the translocations Therefore, these genes will be promising candidate genes for XLMR Three X chromosomal breakpoints have been mapped to distinct loci on the short proximal arm of the X chromosome indicative for the presence of several genes for MR in this chromosomal segment (*chapter 8*) The fourth breakpoint has been mapped to Xq13 1, and has been analyzed in more detail An alternatively spliced X chromosomal gene, *DXS6673E*, has been identified that is disrupted by the translocation in the 5' untranslated region RT-PCR analysis of *DXS6673E* on total RNA from peripheral blood lymphocytes of the patient, showed that one splice variant is under control of chromosome 13 sequences (*chapter 9*) Future research should reveal the involvement of chromosome 13 in the phenotype of the patient and the involvement of *DXS6673E* in the etiology of XLMR

10. SAMENVATTING

Het doel van deze studie was de isolatie en karakterisatie van genen betrokken bij X gebonden doofheid (DFN3) en bij X chromosomaal overervende vormen van niet-syndromale mentale retardatie met behulp van positionele klonering. Ten eerste hebben we het kritieke gebied voor DFN3 en niet-syndromale XLMR gedetailleerd in kaart gebracht en zo mogelijk verder begrensd door de molekulaire karakterisatie van X chromosomale deleties in patienten met CHM, MR of DFN3, of een combinatie van deze ziekten (*hoofdstuk 4 en 5*). Deze karakterisatie van het DFN3 gebied, samen met de bevinding dat het muize *Pou3f4* gen gelokaliseerd is in een X chromosomaal gebied dat homoloog is aan het humane Xq13-q21 segment en dat de rattehomoloog *RHS2* in embryonale stadia van het oor tot expressie komt, resulteerde uiteindelijk in de identificatie van verschillende mutaties in het humane *POU3F4* gen in patienten met DFN3 (*hoofdstuk 6*). Sommige patienten hebben echter chromosomale afwijkingen buiten het gen zelf. Een zo'n afwijking, een paracentrische inversie/duplicatie is nauwkeurig gekarakteriseerd (*hoofdstuk 7*). Deze chromosomale afwijkingen wijzen erop dat er zich mogelijk een nog niet geidentificeerd exon of regulatoire sequenties centromeer van *POU3F4* bevinden. De doofheid in deze patienten kan mogelijk ook nog verklaard worden door een positie effect dat de *POU3F4* expressie beinvloedt, of door de aanwezigheid van een tweede gen in dit chromosomale segment dat een rol speelt bij de binnenoorontwikkeling.

Tevens hebben we met behulp van fluorescentie *in situ* hybridisatie de X chromosomale breukpunten van vier gebalanceerde X,autosoom translocaties in mentaal geretardeerde vrouwen nauwkeurig gelocaliseerd (*hoofdstukken 8 en 9*). Wanneer een X chromosomaal gen door de translocatie verstoord wordt, kunnen deze chromosomale afwijkingen als gevolg van de preferentiele X inactivatie van het normale X chromosoom in cellen van deze patienten, leiden tot een functionele nullisomie voor dit gen. Dergelijke genen zijn dan interessante kandidaat genen voor MR. Drie breukpunten hebben we nauwkeurig kunnen lokaliseren in drie verschillende segmenten van de proximale korte arm van het X chromosoom. Dit suggereert dat meerdere genen op de proximale korte arm van het X chromosoom betrokken zijn bij MR (*hoofdstuk 8*). Het vierde breukpunt, in Xq13.1, is nauwkeuriger geanalyseerd. Een X chromosomaal gen werd geidentificeerd dat onderhevig is aan alternatieve splicing en waarvan een van de splice varianten wordt verstoord door de translocatie. De expressie van deze variant blijkt in de patient onder controle te staan van chromosoom 13 sequenties (*hoofdstuk 9*). Nader onderzoek zal moeten uitwijzen wat de rol is van dit gen bij XLMR.

11. DANKWOORD

Vrijwel elk proefschrift dat ik onder ogen heb gekregen wordt ingeleid of afgesloten met een dankwoord waarin recht wordt gedaan aan de inspanningen van degenen die de promovendus met raad en daad terzijde stonden en op zijn of haar weg begeleidden naar de dag van de promotie Ik heb de indruk dat dit onderdeel samen met het curriculum en de stellingen, het meest gelezen deel van het hele proefschrift is Vanuit die gedachte vraag ik mij wel eens af of het proefschrift niet beter beperkt kan worden tot deze drie onderdelen, met als appendix de geproduceerde artikelen Hoewel dit afbreuk zal doen aan de wetenschappelijke waarde, doet het recht aan het uiteindelijke gebruik Geheel in navolging van het voorafgaande wil ook ik hier van de gelegenheid gebruik maken enige mensen te bedanken

Een promotie-onderzoek vereist veel doorzettings vermogen niet alleen van de promovendus, maar ook van de begeleiders Ik ben Hans-Hilger Ropers en Frans Cremers dan ook dankbaar voor het vertrouwen dat ze in mij hebben gesteld Ik waardeer ten zeerste hun nauwe betrokkenheid bij de vorderingen tijdens mijn promotie-onderzoek en mijn ontwikkeling tot zelfstandig onderzoeker Naast deze gedegen ondersteuning is er nog een belangrijk aspect dat heeft bijgedragen aan de vorming van dit proefschrift Dat aspect wordt bepaald door de mensen binnen het instituut die gezamenlijk het begrip 'plezier in het werk' tot een dagelijkse realiteit maakten De nauwe samenwerking met de verschillende disciplines binnen het Antropogenetisch Instituut kwam goed tot uiting tijdens het maandelijkse XLMR-overleg Daarbij heeft de goede steer op het laboratorium DNA-research veel bijgedragen aan mijn enthousiasme voor de humane genetica Ik wil met name Irene Huber, Inge Scholten, Yvette de Kok en Susann Schweiger bedanken voor de prettige samenwerking Voor de enorme inzet van Dorien van de Pol en Saskia van der Velde-Visser voor het reilen en zeilen van het lab heb ik veel bewondering Ik bewaar goede herinneringen aan deze tijd

Mijn ouders ben ik dankbaar voor hun steun die zij mij altijd hebben gegeven Op hen kan ik altijd rekenen Tenslotte ben ik Pauline veel dank verschuldigd Zij weet mij altijd op de juiste momenten te motiveren en te temperen Haar bijdrage aan de totstandkoming van dit proefschrift is groot

12. CURRICULUM VITAE

Silvère van der Maarel werd op 9 april 1967 in Beverwijk geboren. Na het behalen van zijn Gymnasium β diploma aan het Gymnasium Felisenum te Velsen in 1985, studeerde hij Molekulaire Wetenschappen aan de Landbouw Universiteit in Wageningen. Tijdens zijn eerste 6-maands afstudeervak bij de vakgroep Erfelijkheidsleer onder leiding van Prof. Dr. Christa Heyting kwam hij in aanraking met de molekulaire genetica. Doel van het onderzoek was de karakterisering van genen betrokken bij het synaptonemale complex bij de rat. Vervolgens volgde hij een 6-maands afstudeervak in het laboratorium Procreatie van het Academisch Medisch Centrum Amsterdam bij Dr. Ruud Schutgens. Zijn onderzoek aldaar was gericht op de ontwikkeling van een biochemische diagnostiek voor Neuronale Ceroid Lipofuscinose. Ter afronding van zijn studie liep hij 9 maanden stage bij het Virginia Mason Research Center in Seattle onder leiding van Dr. Eric Milner alwaar hij het humane globuline V_H4 repertoire onderzocht. In augustus 1991 studeerde hij af in Wageningen.

Van 1991 tot 1995 was hij als AIO verbonden aan de Katholieke Universiteit Nijmegen bij de afdeling Antropogenetica waar hij onder leiding van Prof. Dr. Hans-Hilger Ropers en Dr. Frans Cremers het in dit proefschrift beschreven onderzoek verrichtte. In aansluiting daarop heeft hij dit onderzoek bij het Max-Planck-Institut für Molekulare Genetik in Berlijn onder leiding van Prof. Dr. Hans-Hilger Ropers voortgezet. Tevens was hij betrokken bij het aldaar geïnitieerde genoom projekt. Thans is hij verbonden aan de afdeling Antropogenetica van de Rijksuniversiteit Leiden onder leiding van Prof. Dr. Rune Frants. Zijn onderzoek aldaar richt zich op de genetische oorzaken van fascioscapulohumerale spierdystrophie.

Hij is gehuwd met de landschapsarchitecte Pauline Hartman.

13. LIST OF PUBLICATIONS

Sasso, E H , Willems van Dijk, K , Bull, A , van der Maarel, S M and Milner, E C (1992) VH genes in tandem array comprise a repeated germline motif *J Immunol* **149:** 1230-1236

van der Maarel, S M , Willems van Dijk, K , Alexander, C M , Sasso, E H , Bull, A and Milner, E C B (1993) Chromosomal organization of the human V_H4 gene family *J Immunol* **150:** 2858-2868

Huber, I , Bitner-Glindzicz, M , de Kok, Y J M , van der Maarel, S M , Ishikawa-Brush, Y , Monaco, A P , Robinson, D , Malcolm, S , Pembrey, M E , Brunner, H G , Cremers, F P M and Ropers, H (1994) X-linked mixed deafness (DFN3) cloning and characterization of the critical region allows the identification of novel microdeletions *Hum Mol Genet* **3:** 1151-1154

Rohme, D , Siden, T , van der Maarel, S M , Cremers, F P M , Tantravahi, U , Marinoni, J , Ropers, H and Schwartz, C E (1994) Radiation hybrids for the proximal long arm of the X chromosome and their use in the derivation of an ordered set of cosmid markers from a defined subregion in proximal Xq13 1 *Somat Cell Mol Genet* **20:** 1-10

de Kok, Y J M , van der Maarel, S M , Bitner-Glindzicz, M , Huber, I , Monaco, A P , Malcolm, S Pembrey M E , Ropers, H and Cremers, F P M (1995) Association between X-linked mixed deafness and mutations in the POU domain gene POU3F4 *Science* **267:** 685-688

Bitner-Glindzicz, M , Turnpenny, P , Hoglund, P , Kaariainen, H , Sankila, E , van der Maarel, S M , de Kok, Y J M , Ropers, H , Cremers, F P M , Pembrey, M and Malcolm, S (1995) Further mutations in *brain 4* clarify the phenotype in the X-linked deafness, DFN3 *Hum Mol Genet* **4:** 1467-1469

de Kok, Y J , Merkx, G F , van der Maarel, S M , Huber, I , Malcolm, S , Ropers, H - H and Cremers, F P M (1995) A duplication/paracentric inversion associated with familial X-linked deafness (DFN3) suggests the presence of a regulatory element more than 400 kb upstream of the POU3F4 gene *Hum Mol Genet* **4:** 2145-2150

van der Maarel, S M , Scholten, I H J M , Maat-Kievit, J A , Huber, I , de Kok, Y J M , de Wijs, I , van de Pol, T J R , van Bokhoven, H , den Dunnen, J T , van Ommen, G J B , Philippe, C , Monaco, A P , Smeets, H J M , Ropers, H and Cremers, F P M (1995) Yeast artificial chromosome cloning of the Xq13 3-q21 31 region and fine mapping of a deletion associated with choroideremia and nonspecific mental retardation *Eur J Hum Genet* **3:** 207-218

van der Maarel, S M , Scholten, I H J M , Huber, I , Philippe, C , Suijkerbuijk, R F , Gilgenkrantz, S , Kere, J , Cremers, F P M and Ropers, H -H (1996) Cloning and characterization of *DXS6673E*, a candidate gene for X-linked mental retardation in Xq13 1 *Hum Mol Genet* **5:** 887-897

van der Maarel, S M , Schweiger, S , Fryns, J -P, Tommerup, N , Kingsley, K , Olde Weghuis, D , Berger, W , Roest Crollius, H , Cremers, F P M and Ropers, H -H Fine mapping of three X chromosomal breakpoints associated with mental retardation evidence for heterogeneity in proximal Xp (Submitted)

STELLINGEN

BEHORENDE BIJ HET PROEFSCHRIFT "CLONING OF A GENE FOR X-LINKED DEAFNESS (DFN3) AND A CANDIDATE GENE FOR X-LINKED MENTAL RETARDATION"

The association of transcriptional regulators with mammalian position effect variegation may reflect the strict stoichiometry that is required for the interactions in which they participate (Engelkamp, D. and van Heyningen, V. (1996) *Curr. Opin Genet Dev.*, **6**, 334-342).

POU3F4 may play a critical role in primary ovarian failure in patients with Xq13-q21 rearrangements (this thesis).

Differentiation of the inner ear in mammals is controlled by the neural tube (Deol, M S., (1966) *Nature*, **209**, 219-220).

Because each member of the class III POU domain gene family exhibits a distinct, yet overlapping, pattern of expression in the developing and mature nervous system, it is tempting to speculate that combinatorial codes of specific class III POU proteins are responsible for determining specific neuronal phenotypes (Alvarez-Bolado, G., *et al.* (1995) *J. Comp Neurol* , **355**, 237-295 and Schonemann, M D., *et al* (1995). *Gene. Dev.*, **9**, 3122-3135).

Strandstoelen op de zonnige zuidcorridor van het Radboudziekenhuis zouden het verblijf van patienten in het ziekenhuis kunnen verkorten.

Het verschil tussen het aankopen van relatief weinig kunst en het ophangen van relatief veel wandversiering is de kunst van het verstand hebben van kunst.

Het carpoolbeleid van het AZN is een poel van ongemak voor jonge ambitieuze wetenschappers die doorgaans langer doorgaan.

A clean bench is a sign of a sick mind (anonymous).

Nijmegen, 18 februari 1997
Silvère van der Maarel

ISBN 90-9010279-5